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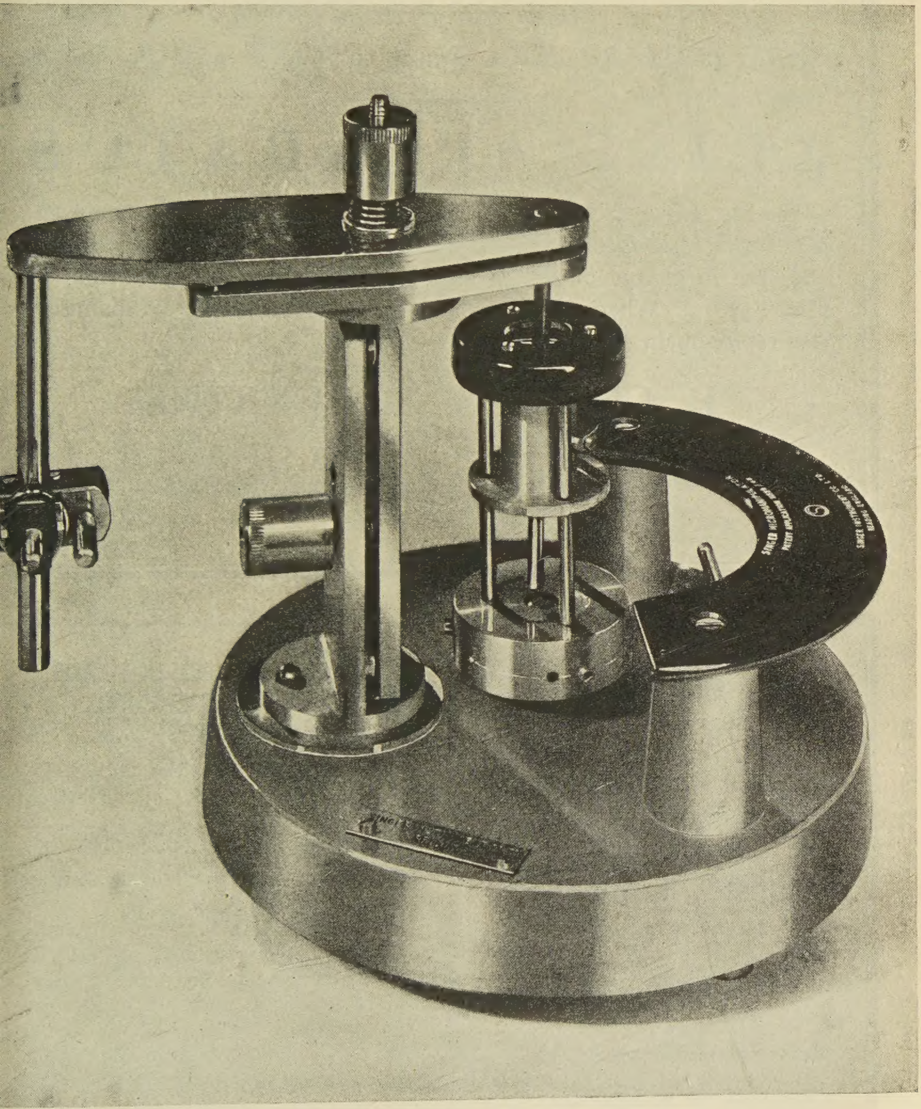

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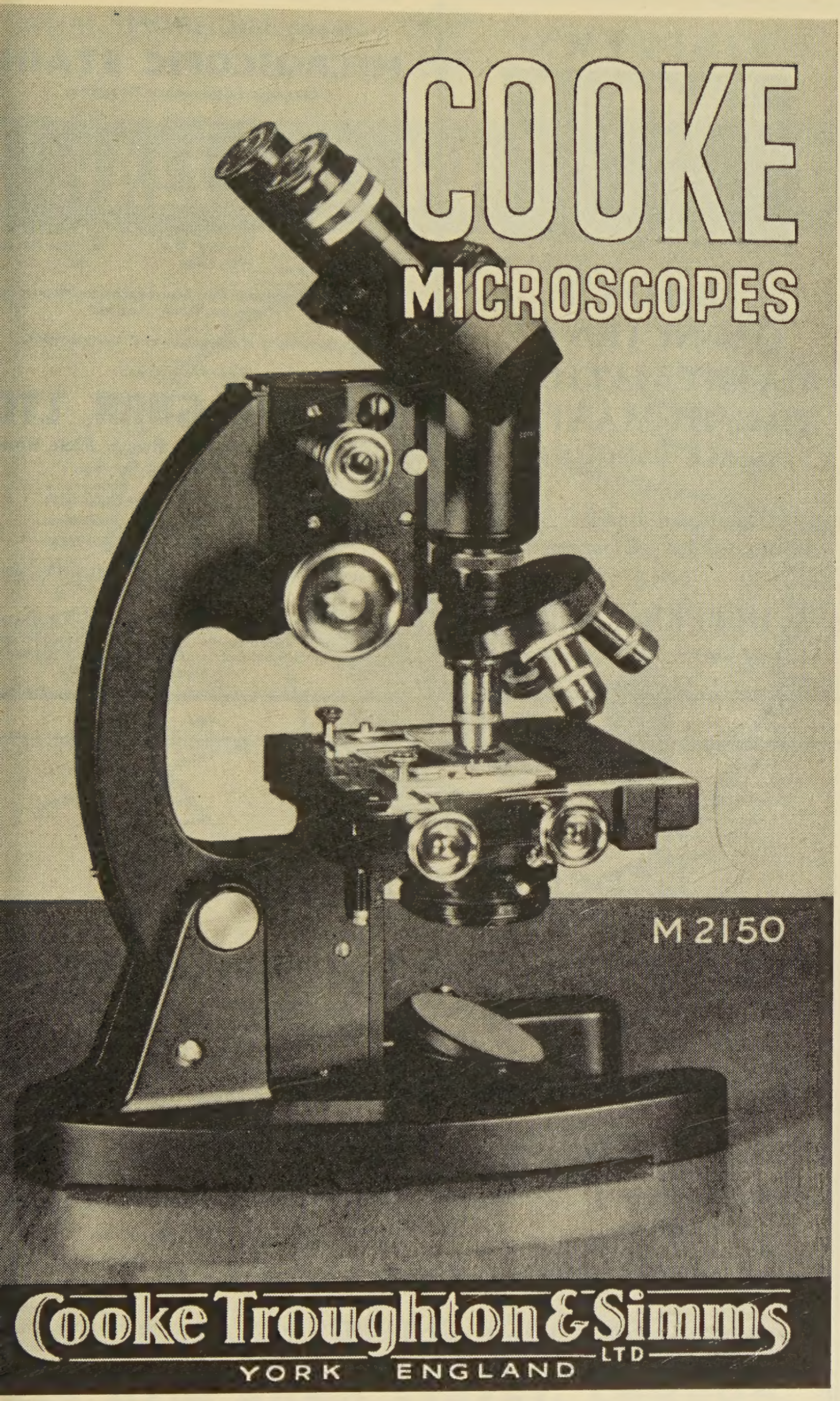
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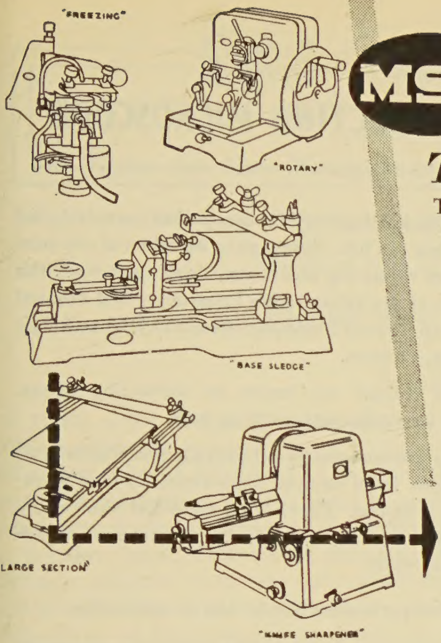

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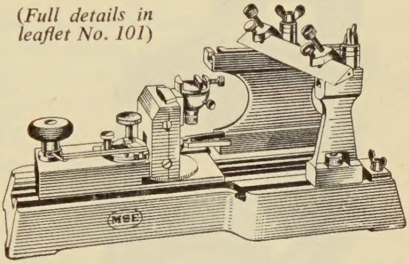

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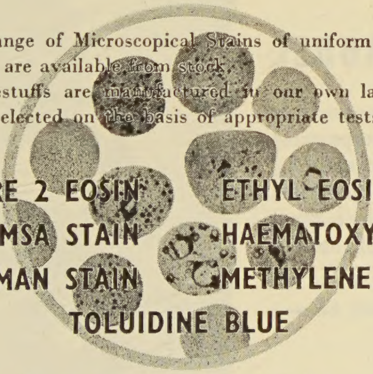

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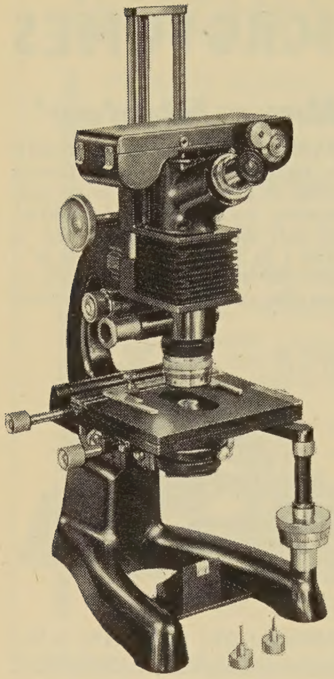

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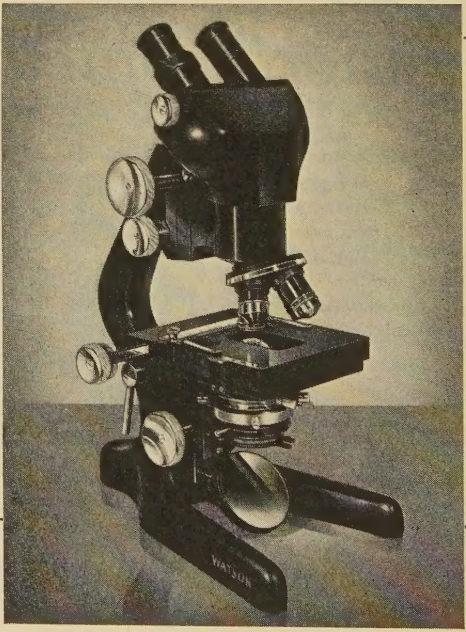

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A Note on the Nematocysts of *Corynactis viridis*

By L. E. R. PICKEN

(*Fellow of Jesus College, Cambridge*)

With two plates (figs. 1 and 2)

SUMMARY

A study of the change in arrangement of the barbs on the thread of the large holorichous glutinant nematocysts of *Corynactis viridis* during discharge shows that the vall of the tubular thread undergoes anisometric dilatation. Together with observaions on the dilatation of the thread in capsules ruptured in an aqueous medium, this trongly suggests that discharge of the thread is due to an intrinsic swelling mechanism: he tubular thread itself swells anisometrically. Though the intrinsic mechanism of lischarge operates at the tip only of the discharging thread, swelling of the capsular luid and elasticity of the capsule-wall must also play a part in normal discharge. While t has not as yet been possible to determine the optical characteristics of the wall of the ndischarged thread (because of the strong birefringence of the barbs), the observed irefringence of the discharged thread indicates an anisotropy of structure such as nisometric swelling would lead us to expect.

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INTRODUCTION

THIS note records observations on the structure and mode of discharge of the large glutinant holotrichous nematocysts of the Jewel Anemone, *Corynactis viridis*. It had been intended to reserve publication until an experinental study of the mechanism of discharge could be made; but views recently

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expressed by Kepner and others (1951) are so much at variance with observations on *Corynactis* and the conclusions to be drawn from them, that a preliminary account seems desirable. Attention was first directed to nematocysts and the problem of their discharge by the realization that the swelling of some part of the emerging thread, postulated by Will (1909, 1914), might be expected to be reflected in the molecular organization of the thread, and this in turn in its optical properties. There was reason to suppose that if the discharge is in part due to an imbibition-mechanism intrinsic to the thread, such that mechanical work is done at the expense of the free-energy of hydration of an anisotropically swelling working-substance, this substance would also prove to be anisotropic in its optical properties. Accordingly, the largest nematocyst readily procurable was chosen and observations were made in 1938 on fresh material, both with the ordinary light-microscope and with the polarizing microscope.

MATERIAL AND METHODS

Material for examination was obtained by teasing out an entire anemone in a few drops of sea water on a microscope slide and covering with a No. 0 coverslip. All stages of discharge were observable, particularly in regions of dense mucus, where discharge was found to be partially inhibited. Examination in ordinary light was carried out with ×10 and ×45 achromatic objectives; a ×92, 2 mm. apochromatic objective; a condenser of N.A. 1·40; and a ×10 periplanatic eyepiece, at a draw-tube length of 170 mm. For examination in polarized light, non-polarizing objectives, ×10, ×45, and ×100 (oil immersion); a ×10 eyepiece; and a one-sixteenth wave-length substage compensator were used. Photographs were taken with a Leica camera.

OBSERVATIONS

Fig. 1, A, shows that there can be no doubt that the barbed thread is in existence before discharge in this nematocyst, which is about 10 times the size of the penetrant stenoteles of *Hydra* (the object of the studies and theories of Kepner with others). Nor can there be any question but that the pre-existing thread is attached to the capsule at a hollow, nipple-like prominence at one pole of the capsule. There is here no 'shaft' and 'spinneret' to be everted, as interpreted by Kepner with others, but a continuous thread is attached at one end to the wall of the capsule. In the partially discharged capsule, one can see that the undischarged thread passes up the lumen of the discharged thread (fig. 1, B and C; fig. 2, A, B, and C); that is to say, it is not formed at a 'spinneret', nor is the undischarged thread an internal filament undergoing 'liquefaction' to form, with a 'capsular matrix', a 'magma of liquefied matrix and liquefied internal filament' (Kepner with others, 1951). What one sees of the undischarged thread is in fact the closely packed barbs, inwardly directed from the wall of the undischarged tube, so as to occlude the lumen completely. Careful focusing shows that the bases of the barbs (apparently circular in cross-section, but see Robson (1953, p. 234)) are in close hexagonal packing (fig. 2

B and C). The image of the close-packed barbs is so complex, that any statement, based on light-microscope observations, about the presence or absence of a membrane on which the bases of the barbs are inserted in the undischarged condition is probably valueless. Since the barbs cohere, however, they are presumably held together by something. They might indeed be stuck together laterally, but their appearance and subsequent behaviour do not suggest this, and it is reasonable to suppose that even before discharge they are inserted on a coherent membrane. In atrichous glutinants, it will be remembered, a tube is present even though barbs are absent.

Fig. 1, B, shows clearly the continuity between discharged thread and capsule-wall. Remarkably enough, the region of continuity is that between the capsule-wall and the hollow, nipple-like 'operculum' already referred to. From the fact of this continuity and from the observation that although many hundreds of discharges have been witnessed no casting-off of an operculum has been seen, it seems reasonable to conclude that there is no operculum in this particular type of nematocyst. The undischarged thread appears to be attached at the centre of the domed, hollow 'operculum', and it is suggested that the lumen of the thread is occluded in some way in the resting nematocyst, but that the membranous wall of the thread-tube is continuous with the wall of the dome. When discharge occurs, the occlusion is removed, and the thread emerges through the now open 'operculum', still continuous with the wall of the dome and, through this, with the wall of the capsule.

If we now examine the discharged thread (fig. 1, B, C, D, E, and F), we see a membranous tube of somewhat greater diameter than the undischarged thread, with barbs inserted on its external surface, no longer in hexagonal packing, but running round the tube in three right-handed helices, 120° out of phase with each other. The distance between the bases of successive barbs in each of these helices is greater than the spacing of the barbs in the undischarged thread; and the distance between any barb and its nearest neighbour in the preceding or succeeding helix is far greater than their spacing in close packing. Such a change in spatial distribution can only have been brought about by an increase in area and, what is more, by an anisometric increase in area, of a surface on which the bases of the barbs were inserted in the undischarged thread. Taking the barbs as points of reference, we can see that a considerable increase in length, as well as an increase in diameter, must have taken place during the discharge of the thread. This is clearly shown in figs. 1, B, and 2, A, where we see a portion of a discharged thread with undischarged thread still inside. Estimating very roughly from the number of barbs in unit length of close-packed, undischarged tube, and the length of tube occupied by the same number in the discharged tube, it appears that the extension in length is about threefold.

Consider now the tip of the discharging thread in a nematocyst in which discharge has not proceeded to completion. It is clear that right up to the tip, the inner thread is still the undischarged thread: the barbs are still in close-hexagonal packing. That is to say, it is unlikely to be something happening in the capsule which leads to change in the dimensions of the undischarged thread; it is more probably something happening at the tip of the discharging thread. At the tip itself (fig. 2, D) there is an extremely fine point, the dart, formed from the most anterior, forwardly directed and projecting barbs of the undischarged thread. The dart is continuous, round its base, with the everted tube, on which the barbs are widely spaced. Clearly, it is at the boundary between internal thread and external tube that the anisometric change in dimensions of the tube must be taking place.

Between crossed Nicols and under low power, capsule and barbs were found to be birefringent, so that the original prediction of anisotropic structure was in some measure confirmed. The capsule wall shows a negative spherulitic cross when lying with its long axis in the plane of the stage (fig. 2, F). Viewed along a normal to the surface, the wall is slightly negatively birefringent with respect to the long axis of the capsule. Since the capsule wall is probably composed of a fibrillar polypeptide (p. 219) this suggests that the chains are lying for the most part with their long axes in the plane of the surface, with a slight tendency to a preferred orientation at right angles to the long axis. From the sharp drop in light intensity immediately within the wall, as seen between crossed Nicols, it is likely that the wall itself is extremely thin (see Mitchison, 1953, and Robson, p. 234).

At a magnification of × 100, it can be seen that the birefringence of the undischarged thread is mostly located in the barbs. If the long axis of the undischarged thread lies in the plane of polarization, the barbs to right and left are bright while the mid-line is dark. This is because the barbs are inserted at an angle of approximately 45° to the long axis of the thread (fig. 2, G). By shifting the compensator slightly, the brightness of one set of barbs can be enhanced, while the other is compensated to blackness (fig. 2, H and I). At a higher magnification (× 450), and in the everted tube, it can be seen that each barb is positively birefringent with respect to its long axis. When viewed along this axis, the barb is not isotropic, perhaps because it has a broad base and is slightly curved, like a rose-thorn (see electron-micrographs in Robson, 1953). In fig. 2, F, one set of barbs is in the addition position, the other in subtraction.

Between crossed Nicols, barbs and capsule are both so bright in the undischarged nematocyst that it is not possible to see what, if any, is the contribution of the membrane on which the barbs are inserted. In the discharged

---

FIG. 1 (plate). A, undischarged holotrichous isorhiza of *Corynactis viridis*, showing regular arrangement of barbs on undischarged thread.

B, partly discharged nematocyst showing (1) change in spacing of barbs between undischarged and discharged condition; (2) three helices of barbs; (3) continuity of discharged tube with capsule-wall.

C, arrested discharge. Undischarged thread extends to the dart. Traces of hexagonal pattern of barbs in undischarged thread are visible.

D, E, and F, fully discharged thread at three different levels of focus—upper, middle, and lower.

hread, however, though the birefringence of the tube-wall is weaker than that
f the barbs, it can be seen that the tube itself is faintly and positively bire-
ringent with respect to its length. This can clearly be seen at the base of the
ully discharged thread, in the region where barbs do not confuse the picture.
he discharged tube itself is seen in compensation in fig. 2, E. Accordingly, the
ube exhibits not merely membrane-birefringence, but also a tendency to
rientation of its substance in the plane of the membrane and parallel to the
ube-axis. The tube, like the capsule, is not isotropic when viewed along a
ormal to the surface, but unlike the capsule it is positively birefringent with
espect to its length.

Observation on nematocysts of *Hydra* showed that the butt of the undis-
harged penetrant as well as the terminal portion of the discharged thread
re positively birefringent with respect to length—as is also the undischarged
nd discharged tube of the glutinants. The coiled and undischarged tube of the
olvent showed a negative cross, brighter than the capsule-cross, indicating
hat the greatest refractive index is parallel to the long axis of the undis-
harged tube. The small diameter of the thread in those types where barbs are
ither small or absent makes it impossible to distinguish (even under oil-im-
ersion) between membrane-birefringence (optical axis normal to the sur-
ace of the tube) and a higher degree of orientation (optical axis parallel to the
ong axis of the tube).

DISCUSSION

1. *Theories of the mechanism of discharge*

Of the various authors who have advanced theories of the mechanism of
ematocyst-discharge which do not invoke activity of the nematoblast itself
r of the surrounding tissue (see Weill's admirable summary, 1934), a majority
ave favoured those in which elastic contraction of the capsule-wall supplies
he energy necessary for the extrusion of the thread. Rather fewer authors
ave favoured theories dependent on swelling of the capsular contents, and
nly a few have been in favour of an active dilatation of the thread itself,
ither as a result of an inherent 'élasticité dilatante' (Weill) or as a result of
welling (by hydration) of part of its substance.

FIG. 2 (plate). A, partly discharged, showing change in spacing of barbs.
B, arrested discharge; traces of hexagonal pattern of barbs in undischarged thread.
C, hexagonal pattern of barbs on undischarged thread.
D, arrested discharge; dart shown. Note helices continued to tip; tendency for discharged
be to be thrown into a helix.
E, fully discharged thread between crossed Nicols with slight compensation; some barbs
right, others dark; tube itself slightly birefringent.
F, capsule between crossed Nicols; spherulitic cross; 'two-layered' wall in three quadrants
ue to differences in focus.
G, arrested discharge, between crossed Nicols. Undischarged barbs bright in two quadrants;
extinction parallel to plane of polarizer.
H, same specimen; brightness of one set of barbs compensated to blackness; other set
right.
I, same specimen, compensation reversed.

That the capsule-wall is not a rigid membrane, but can be distended reversibly and, by its resistance to distension, may maintain a positive intracapsular pressure is clear from the experiments of Weill, among others. But it is also clear from Weill's review of a wide range of nematocyst types, first, that by no means in all cases does the capsule contract at discharge, and secondly, that the final volume of discharged capsule and emerged thread together is often (and indeed usually) greater than the initial volume of the undischarged nematocyst. With respect to volume changes of the whole system (capsule+thread), Weill concluded that there is no definite relation between discharge and volume changes. (Iwanzoff, 1896, had already rejected capsular contractility as the decisive factor in discharge, on the grounds that the capsule does not always decrease in volume.) Particularly impressive as evidence against the contractility hypothesis are Weill's examples of the nematocysts of *Diphyes* and *Forskalia* (Siphonophora) (his 'astomocnides'), where the evaginated tube itself is larger in volume than the discharged capsule, and the total volume is greater than the initial volume, though the capsule itself may have contracted by 20–30 per cent. during discharge.

Iwanzoff (1896) was originally led to the view that the swelling of the capsule contents must be of primary importance by the many instances in which the final volume of capsule+thread together is greater than that of the original nematocyst. Observations by Hadži (1907), Weill (1934), and most recently by Jones (1947), have shown that whatever the change in volume of the capsule may be, there can be no doubt that the volume of the intracapsular fluid increases to at least twice its initial value during and after discharge. Hadži (1907) had observed the tip of a discharging nematocyst which happened to lie under an air-bubble, and watched a droplet of greater volume than the capsule emerge from the tip of the thread at the end of discharge, though the capsule itself contracted but slightly and remained full of liquid. This observation was repeated by Weill (1934, p. 159), who showed that the droplet was usually larger than the capsule. Other observations were made on nematocysts of *Anemonia* and *Actinia*, after staining in neutral red at a concentration of 0·02 per cent. in distilled water (Weill, 1934, p. 147); under these conditions, the capsule-contents are intensely stained, and the rate of discharge in the stain is slower than in distilled water. During the ensuing slow discharge the tube is seen to be filled with stained capsular contents and continues to lengthen for some 10 minutes after the beginning of discharge. Though clear regions appear, breaking up the column of stained contents, no stain can be seen to leave capsule or tube during this period. At the moment when discharge is complete, however, a droplet of stained material begins to form at the tip of the thread. From the movement of unstained zones in the tube, it is clear that the contents are ascending the tube from the capsule both during and after discharge. (These observations have been repeated by Robson.) In the droplet itself, unstained material entering from the tube comes to the surface of the droplet and mixes with the surrounding medium. It would seem, then, that there is a separation of phases as the capsular contents swell—presumably a separation into water-rich and water-poor components, the latter having a high affinity for neutral red. A succession of such droplets forms, always at the tip, and these become detached when their diameter reaches something over 4 μ. The ascending movement and formation of droplets were observed to continue in one case for an hour and a quarter. Weill calculates that the capsule had discharged at least 500 μ^3 of liquid (that filling the tube) and at least 300 μ^3 more were discharged from the tip of the thread. Accepting a figure of 380 μ^3 for the initial volume of the capsule, the volume of capsular contents must have doubled during and after discharge.

In normal discharge, as Hadži and Weill (and more recently Yanagita, 1943) observed, liquid does not appear at the tip until discharge is finished—as if the increase in volume of capsule-contents were both slow and protracted. But Weill has observed in certain holotrichous isorhizas that the capsule undergoes rapid inflation, perhaps to twice its size, before discharge and then returns to its original (or to a smaller) volume, probably before discharge has finished. This most important observation requires confirmation. Personal observations suggest that the capsule does become slightly plumper immediately before discharge.

A few years before the observations described in this paper were made, Weill had also shown that the thread itself dilates during discharge; he claims indeed that it may double its diameter in the large holotrichous isorhizas of *Corynactis*; this is not confirmed by the photographs collected here. Moreover, he showed that in those nematocysts with an axial body or butt, this often elongates to 2 or 3 times its original length, during discharge, so that the tube not only increases in diameter but in length also. On morphological grounds, therefore, there is every reason to believe in a 'dilating elasticity' (in some sense) of the tube wall. If this dilatation were due to an intrinsic swelling mechanism, the thread should extend whenever it is exposed to the aqueous medium. In the course of the present examination of *Corynactis*, a spirocyst was found, ruptured at the posterior end of the capsule, and with the thread stretched out posteriorly through the hole in the capsule. Will (1909) had also described spirocysts bursting at the posterior end and the fibre shooting out backwards. In these cases, extension of the thread occurred presumably without eversion. Weill, too, describes experiments in which other types of nematocyst have been ruptured in sea water. The thread is said to perform abrupt and violent movements for 20 seconds or so, emerging from the capsule without eversion and finally coming to rest with an increased diameter (Weill, 1934, p. 210). Yet another example is given by Weill in an account of experiments on the anisorhizas of *Diphyes* (*loc. cit.*, p. 238), where the thread clearly lengthens, as well as increasing in diameter, and here also emerges from the capsule without eversion. Parker and van Alstyne (1932) reported, however, that threads from nematocysts of *Physalia*, ground with carborundum powder in a mortar, did not discharge in sea water. The details of this experiment are not given, but a possible reason for non-discharge under these conditions will be mentioned later (p. 220). With the exception of these observations of Parker and van Alstyne, the evidence quoted here (together with the experiments of Robson, p. 231) supports the view that the thread has intrinsic powers of unfolding itself; that is to say, it is not simply dilated and extended by the swelling of the capsular contents.

It is clear from this survey that (1) the capsule wall is reversibly extensible and a rise in intracapsular pressure must result from swelling of the capsular contents, (2) the capsular contents swell to at least twice their original volume, during and after discharge, and (3) the thread possesses the intrinsic property of swelling, and this leads to its dilatation without evagination if the capsule is ruptured in a suitable environment.

Accordingly, each of the groups of authors mentioned at the outset has correctly seized on part of the complete story of discharge. Weill himself has summed up his own views as follows (*loc. cit.*, p. 238): 'the mechanism of devagination is represented by the swelling of the capsular contents under the influence of the endosmosis of water or certain other liquids assisted in some measure by the dilating elasticity of the walls of the tube' (present author's translation). Weill's 'other liquids' are without exception aqueous solutions; and for 'dilating elasticity' we may substitute anisometric swelling. It should not be forgotten that Dujardin (1845) suggested that distension of the capsule by endosomotic swelling of its contents led to rupture, but that the thread emerges 'with elasticity'. Zoja (1890) regarded the condition of tension in the invaginated tube, resulting from 'a great accumulation of molecules' on the internal surface during the period of formation, as the essential factor in discharge. Will's picture of active extension of the thread as a result of intrinsic swelling was undoubtedly correct; but he also claimed to find a discrete, visibly swelling substrate, the 'swelling bands', stainable with methylene blue. These, however, as Weill showed, are absent entirely in some forms of nematocyst (see also Robson, p. 235) and when present cannot be seen to swell.

2. *Interpretation of observations and speculations*

(*a*) *The geometry of discharge*

It is immediately apparent from the photographs of the thread before and after discharge, that the arrangement of the barbs changes during discharge; but it is not easy to determine the nature of this change by inspection. At the outset, it seemed possible that the three helices might arise by an anisometric expansion in area of the surface on which the barbs are initially spaced in close-packed hexagonal array. Models showed that if the radius of the circle associated with a single barb-base is approximately one-tenth of the circumference of the undischarged tube, a set of such circles in close hexagonal array will completely cover the surface of the tube when arranged so that the *a*-axis of the hexagonal array makes an angle of 60° (or 120°) with the long axis of the tube (fig. 3). Under these conditions a line drawn from one barb-base through successive nearest neighbours at an angle of 60° (or 120°) to the long axis of the tube, makes one complete turn of the tube in passing through the bases of six barbs. On such a model, as in the photographs, a maximum of four and a minimum of three longitudinal rows of barbs are visible in surface view. If we now allow the tube to expand to the condition of the discharged tube, three helices appear (fig. 4). The following data (from photographs) have been utilized: the diameter of the tube increases during discharge by about 40 per cent.; the spacing of the barbs (between nearest neighbours in a helix) increases by about 50 per cent.; the angle between the helices and the long axis of the tube is about 30°; and in the expanded thread about fifteen barbs are counted in one turn of the helix, as compared with six barbs in the unexpanded hread. None of these values can be regarded as constant.

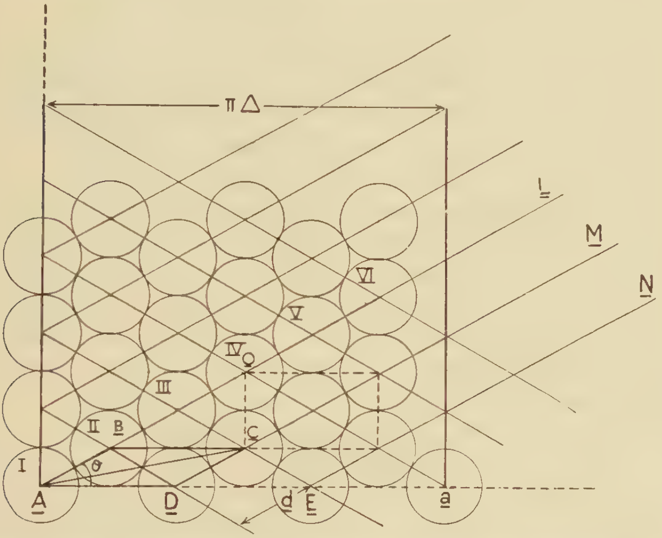


FIG. 3. Diagram showing the disposition of the barbs in close hexagonal array on the surface of he undischarged thread regarded as a plane sheet. $\pi\Delta$ is the circumference of the undischarged thread. The minimum spacing of the barbs is *d*. Nos. I–VI mark the six barbs on line making one complete turn of the thread. *L*, *M*, and *N* are three fundamental helices of nearest neighbours), each making an angle θ with the horizontal, starting from three barbs, *A*, *D*, and *E*. Other lettering is referred to in the text.

An integral number of 'nearest-neighbour' helices can be generated from close hexagonal array by an anisometric expansion whenever the number of ertical (longitudinal) rows of barbs is an even number (2, 4, 6, 8, &c.); and he number of helices generated will be $N/2$, where N is an even number of ertical rows. N appears to be 6 in all barbed nematocysts.

The hexagonal close-packed array corresponds to a special case in the geometry of phyllotaxis (Tait, 1872; see also Thompson, 1942). Consider the oints A, O, and a, in fig. 3 (a coincides with A in the cylindrical case). O is eached in three steps from A along a right-handed helix, and in three steps rom a along a left-handed helix; that is to say, our system of helices on a cylindrical surface is a composite system of whorls, with three fundamenta helices. It follows that the number of fundamental helices will always b equal to the number of steps from A (or from a) to O. In order to extend AC and Oa each by one step, Aa must be extended by $2d \cos 30°$, where d is th

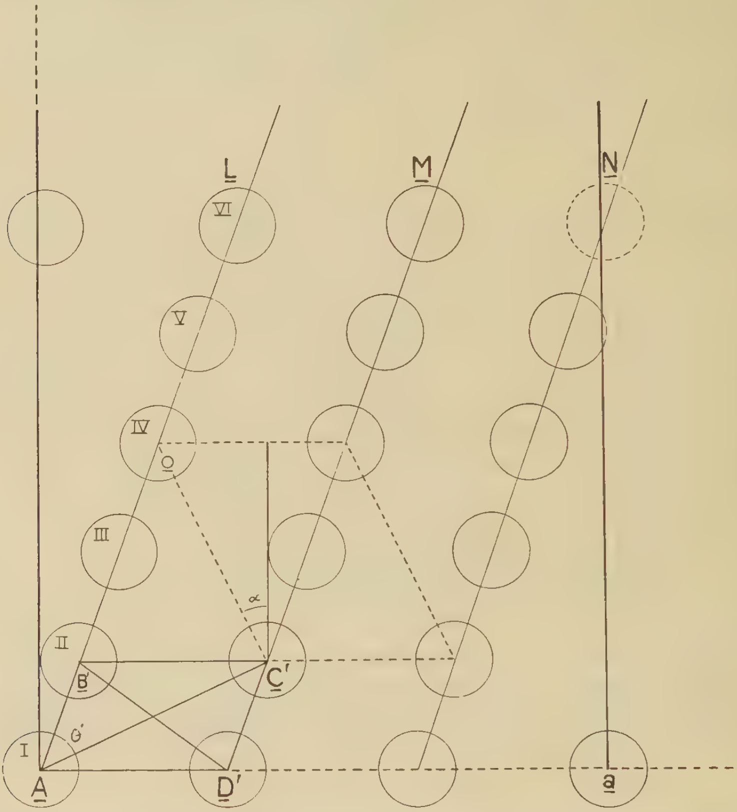


FIG. 4. Diagram showing the disposition of the barbs on the surface of the discharged an expanded thread. θ' is the angle made by the helices L, M, and N with the horizontal. Th parallelogram $ABCD$ in fig. 3 has been transformed to $AB'C'D'$. The dotted rectangle in fig. has been transformed to a dotted parallelogram. In this latter transformation the line CO ha rotated through an angle α.

length of a step. The relation between d (the diameter of the circle associate with one barb) and Δ (the diameter of the nematocyst-tube before dilatation is such that

$$\frac{\pi\Delta}{2d \cos 30^\circ} = n,$$

where n is an integral number of fundamental helices. If we substitute in thi equation a value of 10 for $\pi\Delta$ (see the first paragraph of this section) and o 3 for n, we obtain a value of 1·9 for d. This approximate value of 2 for d agree with the value of 1 for $d/2$, obtained from the model.

In the course of the expansion of the thread, a straight line drawn at random on the unrolled and flattened surface of the undischarged tube will, in general, undergo both a change in length and a change in direction. This is clear from the behaviour of the various lines and quadrilaterals inserted in fig. 3, and shown in the transformed condition in fig. 4. From figs. 3 and 4 it can be seen that lines in three directions, AB, AC, and AD, on the surface of the undischarged tube undergo a change in length of not more than 1·5 times during expansion, while along BD there is a considerable increase in length amounting to about 2·5 times. Along AD there is no change in direction on expansion, and there is only a small change in direction (about 5°) along BD, while lines in the directions AB and AC undergo considerable change in direction. The transformation is epitomized in fig. 5, where the unit-cell, $ABCD$, transforms to $AB'C'D'$. During expansion, the distance AD increases about 1·4 times with no change in direction; AB increases 1·5 times with a change in direction; AC undergoes a small increase in length and a relatively small change in direction; and BD increases 2·4 times in length and undergoes a very small change in direction.

The transformation of a straight line passing through the origin can be regarded as made up of two components, one represented by a change of co-ordinates, and the other by a change from rectangular to oblique axes. In fig. 6, let (X, Y) be the rectangular co-ordinates of a point P on a straight line through the origin O which makes an angle θ with the X-axis. The line OP is transformed to OP', making an angle θ' with the X-axis. The co-ordinates of P' are (mX, nY), referred now to *oblique* axes, obtained from the original rectangular axes by rotating the Y-axis in an anti-clockwise direction through an angle α. If the co-ordinates of P' referred to the original axes are (x, y), then

$$x = mX - nY\sin\alpha, \text{ and } y = nY\cos\alpha$$

so that

$$\cot\theta' = \frac{x}{y} = \frac{mX - nY\sin\alpha}{nY\cos\alpha} = \frac{m}{n}\frac{X}{Y}\sec\alpha - \tan\alpha$$
$$= \frac{m}{n}\cot\theta\sec\alpha - \tan\alpha,$$

m/n, α, are constants for the transformation. Knowing these, the change in direction and the change in length of any line passing through the origin can be determined. The ratio of the coefficients of linear expansion, m/n, seems to be about 1:2.

In drawing figs. 3 and 4, it had been assumed that it is the rows of positive slope (AL, DM, EN) which transform to the helical rows with a positive slope in fig. 4. The correctness of this assumption is shown by the observed counter-clockwise rotation of the advancing tip, to be discussed in the next section.

In the course of the transformation which converts the parallelogram $ABCD$ to the condition $AB'C'D'$, though the length of no side increases by more than 50 per cent., the area increases considerably. If the angle BAD is θ, then $AB \sin \theta$ will be the height of the parallelogram in the undischarged condition. When θ changes to θ', the height becomes $AB' \sin \theta'$. If the length of AB is taken as 1, and AB' as 1·5, the factor for increase in height will be

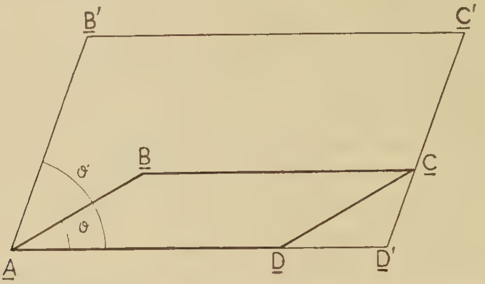


Fig. 5. Diagram epitomizing the transformation: the unit-cell $ABCD$ transforms to $AB'C'D'$.

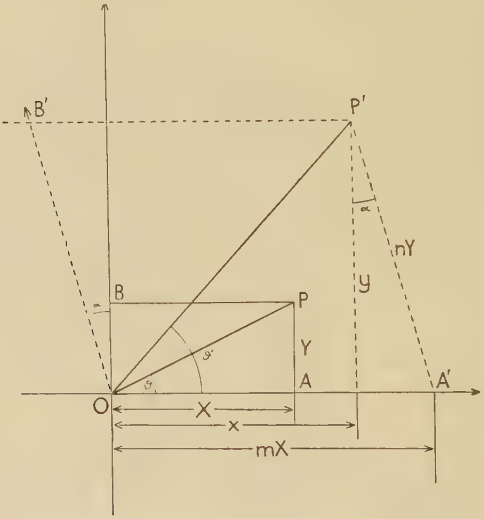
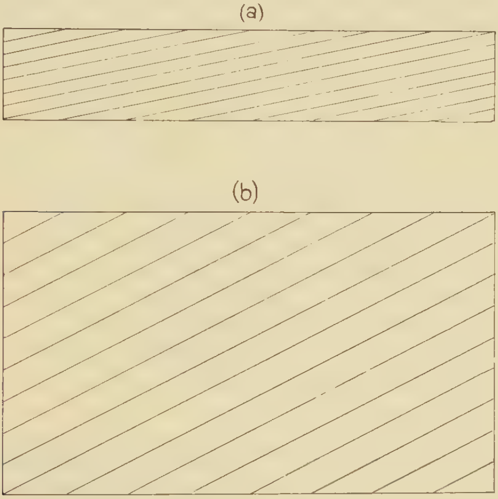


Fig. 6. Diagram illustrating the general case for the transformation of a straight line passing through the origin by a change in co-ordinates and a change from rectangular to oblique axes. Lettering explained in text.

(substituting an approximate value for θ'): 1·5 sin 70°/sin 30°, that is, *c.* 2·8 times. Neglecting the change of AD to AD', the factor for increase in area is the same as for increase in length. An approximately threefold increase in length implies, therefore, at least a threefold increase in area of the tube. Allowing for dilation as well as for shear (as in fig. 5) the increase in area is approximately fourfold.

The transformation of $ABCD$ to $AB'C'D'$ might be expected to occur under the following conditions. Let us suppose that the barbs are inserted on a membrane consisting of parallel chain-molecules or micelles oriented on ight-handed helices making a small angle with the direction *AD*, approxinately parallel to the direction *AC*. Let us further suppose the oriented naterial to be dehydrated and to have an affinity for water; and let it be suffiiently cross-linked to prevent dispersion in water, but not sufficiently crossinked to prevent shear between adjacent helices when water is taken up etween the helices. The observed lengthening of the tube might then be proluced if each helical element of the underlying membrane took up water in uantity sufficient to treble its area. Such a system is shown diagrammatically n fig. 7.



'IG. 7. Diagram showing how the observed lengthening of the tube might be brought about y a trebling of the area between helical elements whose horizontal spacing is fixed: (*a*) undisharged condition; (*b*) discharged condition. The diagram is simplified by neglecting expansion in diameter.

b) Rotation

It is an essential feature of the transformation of the thread from the closeacked to the expanded condition that horizontal lines in fig. 3 remain horizontal n fig. 4, while vertical lines in fig. 3 undergo rotation to the left through n angle α in fig. 4. This implies that the dart, at the tip of the expanding ube, must be rotating in a counter-clockwise direction, in a sense opposite o that of the three helices. In order to simplify two-dimensional representaion, let us first consider only the shearing component of the transformation. 'ig. 8 shows part of two out of the three expanded rows of barbs and their inction with the unexpanded tube. The anterior edge of the tube is repreented by the line *BC*. The circles *A* and *D* are the bases of two members of ne last whorl of barbs to be added to the helices of the expanded tube. From nem, barbs will stand erect, normal to the plane of the diagram. The circles } and *C* are the bases of two out of the three barbs temporarily forming the art. From them, barbs will project forwards, converging on a point on the axis of the tube. Because the line AD in fact lengthens to AD', AD will not coincide with AD' as shown in the diagram. Neglecting for the time being this dilatation of AD, however, we can conveniently represent the transformation in two dimensions. If we suppose that A and D are points fixed in space, then, at the moment of transformation (neglecting dilatation) $ABCD$ will shear to $AB'C'D'$. The effect of this shearing (since A and D are fixed) must be to displace the whole unexpanded array to the right (in the diagram). Since the unexpanded array is in fact on a cylindrical surface, this displacement will take the form of a rotation through an angle whose magnitude can be determined from the horizontal distance between B and the point B'. If this distance is x, and the diameter of the undischarged tube is Δ, the angle of rotation, ω, is given by

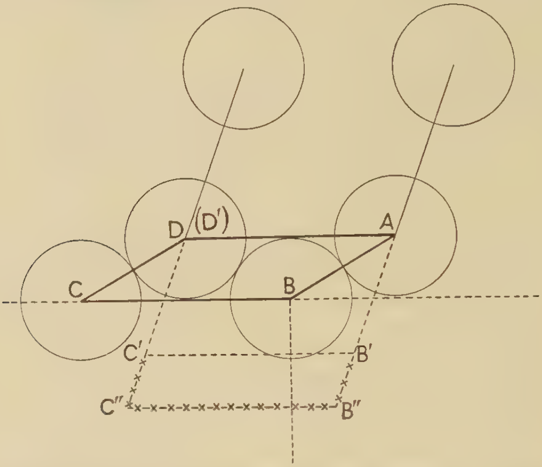
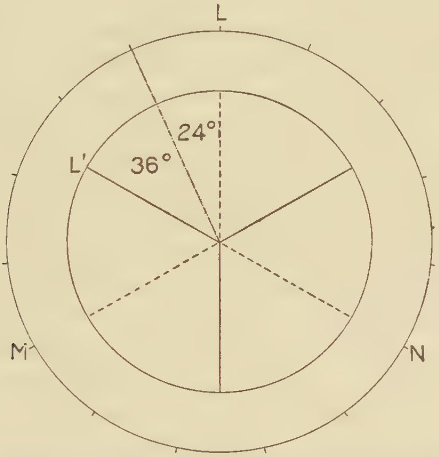


FIG. 8. Diagram showing in two dimensions the transition between unexpanded and expanded barb-series at the advancing tip, during discharge. Explanation in text.

$$\frac{x\,360}{\pi\Delta}$$

From fig. 8, ω would seem to be about 36°. This means that for each barb added to the barb-row the unexpanded tube must rotate through 36°, if we suppose that the expanded tube is not free to rotate, but that the unexpanded tube is free to do so. ($AB''C''D'$ is the final state after dilatation along AB' and $D'C'$.)

A check on this value is obtainable. If we assume that there are fifteen barbs to one turn of the expanded helix, the angle between successive barbs (measured round the circumference of the expanded tube) must in fact be $\frac{360}{15} = 24°$. We can represent the expanded tube, with the fifteen barbs of one turn of the helix in projection, as a circle (fig. 9). Points L, M, and N mark one whorl of barbs (that most remote from the observer), but only the L-series is continued in projection round the circle. Within this circle is drawn a smaller oncentric circle, representing the projection of the undischarged tube. The ifference in diameter between the two circles arises from the dilatation in the irection *AD*, *not* represented in fig. 8. Looking down on the tip of the adancing thread, successive barbs in the *L*-series appear disposed in an antilockwise sequence at intervals of 24° starting from *L*, round the circle. On the nner circle, the three full lines represent the three barbs momentarily forming he dart; the three dotted lines are the next whorl of barbs which will form the art when the full lines have been reflected as the next three barbs in the eries *L*, *M*, and *N*. If *L*, *M*, and *N* have just been laid down on the expanded



IG. 9. Diagram showing the advancing tip of the discharging thread as seen by an observer looking towards the capsule. Explanation in text.

ube, the three barbs of the dart will lie at an angle of 60° to the whorl *L*, *M*, *N*, s shown by the position of the full lines on the inner circle in the diagram. The arb marked *L′* will form the next member of the *L*-series, and it is clear hat in order for it to do so the inner circle must rotate clockwise through 0—24 = 36°, the value obtained graphically in fig. 8. (The exact agreement fortuitous.)

Looking from the capsule towards the advancing tip of the thread, therefore, he still undischarged thread and the dart must be rotating counter-clockwise. his rotation implies, however, that the whole undischarged thread must otate as it is drawn up the lumen of the discharged tube. Since one turn of a elix of close-packed barbs includes six barbs, the addition of five barbs to one xpanded helix will mean a rotation of 5 × 36 = 180° of the undischarged tube. nd since a complete turn of one expanded helix includes fifteen barbs, the ndischarged tube must rotate one and a half times before a sufficient number f barbs have moved into position to form three helical turns, each of fifteen arbs. Since the undischarged thread is coiled like a rope within the capsule, must twist as it is uncoiled, and the rotation of the tip presumably facilitates this uncoiling. This anti-clockwise rotation of the undischarged thread has been confirmed by Robson, p. 231.

(*c*) *The behaviour of the barbs*

As the thread emerges from the capsule, its tip must be constantly formed anew, and from the dart, barbs must be constantly swinging backwards through *c.* 135° to take up their final position on the external surface of the tube. Although this type of nematocyst is a glutinant, this behaviour of the barbs, coupled with the drill-like rotation of the dart, must enhance the water-penetrating power of the tip and will tend to keep it on a straight course. Since the backward swing of each barb through *c.* 135° is combined with rotation through 36°, the path executed by the tip of each barb in turn as it swings out from the dart with the swelling of the membrane must be a portion of a helix.

Eversion of the tube follows necessarily from the mechanical continuity between the rim of the undischarged tube and the rim of the aperture o emergence from the capsule (fig. 1, B). Without such continuity one would suppose that the undischarged tube would lengthen if allowed to swell, but would not evert. This has been confirmed by Robson, p. 232. Continuity between undischarged and discharged thread not only leads to outward rotation of the barbs over the rim of the advancing thread-tube, but also to the undischarged portion of the thread being drawn out of the capsule and up the lumen of the discharged thread.

(*d*) *The thread as an imbibition-mechanism*

If expansion occurs only at the tip (as is suggested by the photographs) only a small fraction of the total volume of swelling material is exposed to the aqueous medium at any moment. Such economy is in accordance with the nature of imbibition reactions. The heat of hydration of highly polymeric substances (such as starch, cellulose, and wool) falls rapidly from fairly high values for the earliest stages of hydration, to low values, as soon as quite a small amount of water has been taken up. This means that if a swelling process is to continue to result in the performance of mechanical work, the total quantity of working substance must be fractionally exposed to hydration in successive instants of time. If a nematocyst thread, isolated from the capsule, is hydrated at all points simultaneously, it will elongate, but it will not evert; nor will it necessarily straighten out (see Robson, p. 232). It might be expected to execute movements comparable to those of an elater (see Ingold, 1939), for a few seconds, but it could not be used as a penetrating device, for example. By disposing the swelling substance as it is disposed in the undischarged thread within the capsule, provision is made for a maximum mechanical effect—the cutter-blade-like action of the barbs—to occur where most effect can be produced, namely, at the tip. In this region, for movement through a distance of a fraction of a micron, corresponding to the first stages of hydration, we may have swelling forces of a very high order. Gurney (1947) has calculated that for spruce at 18° C. in equilibrium with air of 60 per cent. relative humidity and subjected to a tensile stress along the grain, the proportional change in moisture content per lb./sq. in. stress is about $0\cdot1 \times 10^{-6}$ at zero stress. That is to say, a change in tensile stress of 1 lb./sq. in. will produce a change in moisture content of about one part in ten million. Conversely, an increase in moisture content of one part in ten million will generate a pressure change normal to the grain of the order of 1 lb./sq. in. The proportional change in moisture content per lb./sq. in. will change with the absolute water content, but at least this figure shows that relatively enormous forces may be generated by anisometric swelling.

(*e*) *The nature of the working substance*

This leads us to inquire what the working substance may be. Brown (1950) obtained evidence of the presence of disulphide-linkages in the capsule and barbs of these large glutinants of *Corynactis*. Inasmuch as the nematocyst is intracellular and (in some instances at least) ectodermal in origin, it conforms to the conditions of occurrence of true keratins in the animal kingdom. In the fully keratinized (that is, disulphide-linked) condition, the keratins are only capable of taking up small amounts of water, and indeed their functional importance as covering for terrestrial vertebrates is largely due to their impermeability, or at least low permeability, to water (Pryor, 1946). At first sight, a swelling mechanism based on keratin as a working substance would seem singularly ineffective. The work of Longley (1949) and Mercer (1949) has shown, however, that the keratins are laid down as fibrillar proteins in keratinizing cells before any trace of disulphide-linkage or even of *SH*-groups can be detected histochemically, and Mercer (1949) showed that saturated aqueous urea-solutions act strongly on this oriented, but not as yet cross-linked, fibrillar protein. The observations of Brown refer to capsule and close-packed barbs of the undischarged thread; they do not exclude the possibility that the undischarged membrane, to which the barbs are attached, is not completely cross-linked. Since it persists as a membrane after swelling, it must be cross-linked to some extent; but it need not be cross-linked in the same way or to the same extent as a true keratin.

The work of Weill (1934, p. 273) on the development of nematocysts has confirmed that it is the capsule which is first delimited, as a vacuole whose boundary progressively acquires optical definition and stainability; that the thread begins as a tube, growing in from one pole of the capsule, lengthening and coiling within the capsule (the reports of everted tubes seem to be based on artifacts); that this tube is filled with barbs in due course, in barbed types; and that, last of all, the operculum is formed at the pole where the tube grows in. Supposing that the disulphide linkages of the nematocyst-protein develop (as those of hair and skin) subsequently to the orientation of the protein, may we not expect linkage to be carried to completion only in the regions most exposed to the cytoplasm—the external surface of the capsule and the last-formed barbs? Alternatively, the disulphide-linked protein may be a different substance from the thread, secondarily formed, and deposited (after a first wave of synthesis has led to the formation of capsule- and thread-rudiments) over exposed surfaces only: as a wall to the primary vacuole (the capsule), and in the lumen of the thread (as barbs). The deciduous character of the barbs in certain types of nematocyst (Weill, 1934, p. 145) suggests that they are in a sense adventitious—stuck on as an afterthought.

Remembering that thread and capsular fluid both derive from one original vacuole, it is not unreasonable that both should be highly hygroscopic substances. Within the capsule it is reasonable to suppose that there will be equilibrium between thread and contents, both sharing water according to their respective affinities. If the contents also have a high affinity for water (as their swelling suggests) there is no mystery in the thread remaining contracted within the capsule; as Weill points out, whatever its 'dilating elasticity' may be, the thread does not in fact fill the capsule in the resting undischarged condition—it is in no sense mechanically constrained by the capsule.

Capsular fluid and thread may be closely related chemically. A difference between a short, branched chain-structure (for the contents) and a long, unbranched chain-structure (for the thread), for example, would perhaps suffice to account for the difference in texture, even if the two were otherwise chemically closely related. Branching of the molecule would tend to keep the contents liquid, prevent their crystallizing out, and result in their immobilizing a relatively large volume of associated water. We can understand why water (as liquid, or as vapour—as Jones, 1947, has noted) is indispensable, if it is only water molecules that can be incorporated between those of the contents and those of the thread, and so lead to the swelling of both. We can predict (as has been verified for certain types of nematocyst) that hypertonic or non-aqueous media inhibit discharge (see Robson, p. 233). Moreover, the presence of other substances than salts with an affinity for water as high or higher than that of the thread or capsule-fluid should also prevent discharge. It may be the presence of high concentrations of mucus which leads to the frequency of incomplete discharge in smears of *Corynactis* nematocysts, and it may have been the presence of such substances in Parker and van Alstyne's experiment (p. 209) which led to non-extension of the threads set free from capsules ruptured in sea water.

(*f*) *The permeability of the capsule to water*

While it is certain that the capsular fluid takes up water during and after discharge (possibly also immediately before), it is not certain that the capsular wall itself (as opposed, say, to the limited region of attachment of the thread to the capsule) is permeable to water; the evidence is ambiguous. Jones (1947) showed that the capsules of undischarged penetrants of *Hydra* transferred to absolute alcohol from water shrank in 30 minutes by as much as 30 per cent. and he concluded from this, and from the penetration of dyes, that the capsule wall is permeable to water before, during, and after discharge. Yanagita (1943), however, was unable to detect significant shrinkage in nematocysts from acontial filaments of *Diadumena luciae* in 4 M glycerin, 4 M aqueous NaCl, undiluted egg-white or 10 per cent. albumin in M/2 aqueous NaCl buffered at pH 8·3 by $NaHCO_3$. It is important to remember that ionic conditions may be expected to have a profound effect on the swelling properties of capsular fluid and thread, and that some of the observed anomalies may disappear when the system is better defined with respect to ionic composition. Our knowledge of the chemistry of the capsular fluid is of the slightest.

It is possible that water does not penetrate to the interior over the whole of the capsule surface, but only in the region of attachment of the thread. A limited zone of entry would be compatible with Jones's observations on shrinkage of the capsule, or Weill's observations on dye-penetration, as well as with the observation that dilatation of the tube begins in the opercular region.

(*g*) *The relation between the thread and the capsular fluid*

Can we attempt to assess the relative importance of swelling of the contents and swelling of the thread, in discharge? It would seem possible to do so on *a priori* grounds. In the isolated nematocyst only a rise in intracapsular pressure can be invoked to explain rupture in the opercular region. Once the capsule is ruptured, the emerging tip finds itself in a medium in which the thermodynamic activity of water is presumably far higher than in the capsular fluid. Indeed, even though the swelling of the contents may start the discharge by rupturing the opercular region, the presence of capsular fluid with a high affinity for water should prevent discharge taking place in the capsule, even though water is entering the system, provided that the imbibition pressure of the thread is somewhat less than the osmotic pressure of the capsular contents. From appearances such as that in fig. 1, C, it is plain that hydration of the thread is occurring at the tip only; otherwise the barbs in the central thread would not continue to be close packed from capsule to dart.

Meanwhile, the volume of the capsular fluid is increasing, and a steady pressure (since water is incompressible and the capsule-wall is elastic) must assist the forward movement of the tip of the thread (though probably not necessary for this movement), and also its eversion. The last is due to the spatial relations of the end of the thread with the capsule-wall, and to the presence of a positive intracapsular pressure. A series of observations by Weill on the anisorhizas of *Abylopsis* has shown very strikingly that discharge of the thread is over long before the capsule fluid has come to equilibrium with the aqueous medium. In these nematocysts, a small sphere closes the distal end of the resting invaginated tube, and in the course of discharge this spherule eventually obturates the proximal end of the tube. Behind the point of obturation, the tube now dilates until its volume is about equal to that of the capsule itself, indicating that water continues to enter the capsule even though the thread has reached its maximal extension.

The relation between the two parts of the mechanism can be summarized in the following way. For dilatation, the thread requires to take up (judging from the thinness of its wall) an excessively small quantity of water; for maintenance of turgor, however, it requires a volume-increase in the liquid contents of the capsule equal to the increased volume of the thread at each instant of discharge. If the thread were simply a glove-finger everted under pressure, it would (on account of its diameter) offer great resistance to eversion. As Weill has argued, however, as a result of the dilatation of the tube during eversion, the undischarged thread is drawn up a tube of wider bore, lubricated with capsular fluid. Furthermore, the advancing tip of a glove-finger would have no tendency to rotate and hence little lateral or longitudinal stability. It is possible (though so far as is known not suggested by the evidence) that the astomocnides of *Diphyes* and *Forskalia* do not rotate, and that the penetrant mechanism may have evolved from a stage with a non-rotating thread. A slight change in packing of the molecules of the expanding membrane could change its swelling behaviour from a dilatation with increase in diameter or in length only, without rotation, to a dilatation with increase in length as well as in diameter, and with rotation.

Since the lengthening and discharging thread is filled with undischarged thread through half its trajectory, it will have, during half the period of discharge, a greater rigidity than the completely discharged thread. Presumably a penetrant nematocyst has usually penetrated sufficiently for effective action on the prey within a distance of less than half its fully discharged length. Weill pointed out, and photographs of *Corynactis* confirm, that in many nematocysts whose discharge has not proceeded to completion, the undischarged thread and the surrounding tube are thrown into a lax helix. This might well result from slight binding of the undischarged thread within the discharged tube, so that the inner tube does not emerge from the capsule with complete freedom and is slightly under tension.

It has been assumed that the dilatation of the thread is due to hydration of substance laid down in a dehydrated condition during the formation of the nematocyst. This is the simplest assumption and accords with the observed dependence of discharge on the presence of water vapour only, and with the possibility of interrupting discharge by dehydration. It remains, however, an assumption, and though dilatation is undoubtedly associated with the uptake of water-molecules, the observations do not exclude the possibility that, for example, some enzymatic process facilitates an eventual swelling reaction. Experiments by Robson (see p. 233), however, seem to exclude the possibility of an enzymatic reaction playing any part in discharge.

(*h*) *Optical properties and molecular structure of the thread*

Though the undischarged thread is clearly birefringent, it has not been possible to relate optical properties unequivocally with swelling-properties, as postulated at the outset. The reason for this failure is the practical difficulty of determining the optical properties of a tube of diameter as small as the undischarged thread, particularly when barbs are present. The discharged tube, however, is slightly positively birefringent with respect to its length when viewed along a normal to the surface. This can be seen plainly at the proximal end, where it joins the capsule and where barbs are absent. In the undischarged thread, the barbs make it impossible to see the membrane in *Corynactis*, but in volvents of *Hydra* the thread is again positive with respect to its length. These nematocysts are too small, however, for it to be possible to distinguish between birefringence of the edges and of the centre of the undischarged thread.

In the explanation of transformation offered in section (*a*) on p. 210 it is suggested that chain-molecules or micelles in the membrane are oriented with their long axes approximately parallel to the direction *AC* in fig. 4. The choice of this direction was determined by the fact that lines in this direction undergo minimum lengthening during transformation, and it seemed reasonable to suppose that if, as its anisometric swelling suggests, the tube-membrane is an oriented fibrillar aggregate of chain-molecules, this direction should be the chain-direction, since changes in dimensions of fibrillar aggregates on swelling are in general minimal in the chain-direction.

If, however, the oriented chain-molecules are those of a typical keratin-like alpha-polypeptide, the optically slow direction of the aggregate should be parallel to the chain-direction, and the two crossed sets of chains on upper and lower surfaces of the undischarged tube should give a resultant maximum retardation at right-angles to the long axis. That is to say, the centre of the tube should be negatively birefringent with respect to its length, and the sides only should be positively birefringent. When the tube is not more than 2 or 3 μ in diameter, however, it will not be possible to distinguish between brightness of edges and brightness at the centre. This is the position with the volvents of *Hydra*. Up to the present this is the only approximately barbless nematocyst examined between crossed Nicols.

Supposing that *AC* is indeed the chain-direction in the undischarged thread, then even after discharge (fig. 4), the centre of the thread should still be negatively birefringent with respect to its length, because not until the line *AC* makes an angle less than 45° with the long axis will the birefringence of the membrane (viewed along a normal to its surface) become positive with respect to the long axis of the tube. It is possible, however, that the barb-rows indicate the direction of orientation of the chains. The barbs themselves may emerge from the surface of the tube like the fine chitinous hairs from the fibrillar ridges of certain lepidopteran scales, such as the spatulate scales of *Trepsichrois mulciber* (Picken, 1949). If this were so, the centre of the tube should be negatively birefringent in the undischarged condition and positively birefringent after discharge.

A fibrillar aggregate in which chain-molecules or micelles are oriented parallel to the long axis increases in girth but scarcely at all in length (or may even shorten somewhat) on swelling. This is the condition and behaviour of hair, elastoidin fibres and muscle, for example. For a fibrillar aggregate to lengthen on swelling, the chain-orientation must be approximately at right-angles to the length of the fibre. The outer segment of retinal rods lengthens on swelling, as Schmidt (1951) has shown, and in that structure there is reason to suppose that polypeptide chains run at right-angles to the long axis in a series of disk-like lamellae. It would seem that the nematocyst thread is intermediate between these two types: it not only lengthens but also expands somewhat in diameter, and it twists as it lengthens. It is simplest to suppose that this behaviour is due to the swelling elements being inclined at an angle greater than 45° but less than 90° to the long axis of the thread.

The arguments for an intrinsic mechanism put forward here are deductions from observations on the structure and behaviour of the thread. They require for confirmation a demonstration that the thread can discharge in the absence of any possible transmission of fluid pressure from the capsule, and also a demonstration that eversion is dependent on mechanical continuity between the rim of the emergence pore and the end of the undischarged thread. Evidence of this kind has been obtained by Miss E. A. Robson and is described in a subsequent note (p. 229). Whether or not the dilatation of a nematocyst thread can be reversed experimentally is a matter for future investigation.

3. *The development of nematocysts*

Some points in Weill's analysis of development are suggestive. The fact that a hollow tube grows into the primary vacuole implies, perhaps, that some insoluble substance continues to be synthesized and deposited at such a rate that the area of the vacuole surface (on which at first it is deposited) is not sufficient to accommodate it. The invagination of a tube, rather than the centrifugal outgrowth of a tubular extension from the vacuole, may be due to the fact that the vacuole surface is tending to dilate slightly with age, or that the tube is tending to contract in diameter as it forms. The occurrence of ingrowth at a pole of the ellipsoidal capsule need not surprise us, since there the curvature of the surface (and hence molecular strain) is greatest. Nor need the fact that ingrowth occurs at one pole only be regarded as surprising: we have sufficient examples of the polarization of cell-function from other types of cell. Any small asymmetry of the nematoblast may provide a disturbance sufficient to determine both axial (rather than radial) symmetry and polar dissimilarity—as Turing (1952) has argued in relation to a morphogenetic problem of higher order, namely gastrulation.

The distribution of the barbs in close hexagonal array in the undischarged thread strongly suggests that conditions of packing—of maximal utilization of space—have determined their arrangement. Some measure of regularity in distribution is not surprising, for each barb (as its birefringence shows) is an ordered fibrillar aggregate, and one would expect the 'nuclei' of the developing barbs to compete for barb-substance like crystal nuclei in their mother-liquor. The regular distribution of the barbs in space might then be comparable to that of the longitudinal ridges and transverse rungs in insect scales (Picken, 1949). Considering, for a moment, the geometry of barb-arrangement in terms of the stationary chemical wave theory of Turing (1952), the 'chemical wave-length' would seem to be not $\pi\Delta/n$, where Δ is the diameter of the undischarged tube and n is the number of barbs in a whorl (as proposed by Turing for Woodruff), but d, that is, the spacing of the barbs along the a-axis of the hexagonal array. This may be due to the essentially two-dimensional character of the problem, unlike the examples of ring or filament considered by Turing.

The entire nematocyst may be interpreted as a record in time of changes in rates of synthesis and deposition of the nematocyst protein; isorhizas being generated if there is but one point of inflection in the curve: $\frac{\text{rate of deposition}}{\text{time}}$, anisorhizas if there are two points of inflection, before the deposition of the barbs begins. The deposition of barbs itself would correspond to a second or third inflection associated with a marked rise in rate of deposition. In a form such as *Hydra*, then, different elements of the cnidome would correspond to four different curves of $\frac{\text{rate of deposition}}{\text{time}}$, associated with four different sizes of nematoblast. It is remarkable and suggestive that within each of the giant battery mother-cells of *Hydra* the nematoblasts come to arrange themselves (as shown by von Gelei, 1927) approximately in concentric zones about a single central stenotele, as if the series: stenotele, holotrichous isorhiza, atrichous isorhiza and desmoneme, corresponded to a descending 'metabolic' gradient. One is reminded of the gradient in 'urate'-production about a macrotrichium in the insect cuticle, and of the studies and interpretation of plaque-organization by Wigglesworth (1933–4; 1940). It is to be remembered, however, that the nematoblasts only reach this arrangement after migration.

Regarding the migration of nematocysts, it is perhaps significant that 'foreign' cells (amoebocytes in *Microstomum*, for example, and in aeolids) also take possession of capsules (set free by digestion), and migrate with them to the surface of a new host. Conceivably, the migration to the surface might be imposed on the cell by the presence of the capsule, in the sense that the cell (whether nematoblast or amoebocyte) is compelled by the presence of the capsule—by its surface properties, primarily—to wander centrifugally as far as it can, until in fact that portion of the cell surface to which the capsule is closely applied is presented to the aqueous medium. The final orientation of the capsule is what one might expect, if the 'wettability' of capsule by cytoplasm had become progressively less during development. The capsule will then tend to become applied to the surface membrane and this may lead to the polarized migration of the cell. The nematoblast (or amoebocyte) may be tending to extrude the nematocyst and indeed usually does so after its discharge.

4. *The evolution of nematocysts*

Something resembling the nematocyst of the Cnidaria, in construction or action, has appeared several times in evolution, in widely separated groups (for a review see Martin, 1914): in the cnidocysts of Dinoflagellata, where natural discharge and function are unknown, but where discharge appears to be associated with eversion; in the pole-capsules of Cnidosporidea, where eversion occurs; in the stinging capsules of the proboscis of certain Nemertini (*Micrura* and *Cerebratulus*, for example); and in the various types of trichocyst in Euciliata, where in some cases at least eversion does not occur, but a strongly asymmetric, longitudinal swelling of the contents, or of the entire trichocyst, takes place (Dragesco, 1952). A common property of dramatic hydration may be shared by all these, with the explosive corpuscles of certain flagellate groups (Cryptomonadina, Chloromonadina); with the cyst-forming corpuscles of ciliates in general (Kahl, 1935); and through these with mucus-producing cells of all kinds. The virtue of mucus itself (see Carter, 1946) is precisely that, thanks to its long, branched chain-structure and abundance of hydrophilic dipole groups (such as $—NH.CO.CH_3$, $—NH_2$, $—COOH$), it has a high affinity for water, and sufficient coherence (insolubility) not to disperse rapidly under normal environmental conditions, and so is able to form a lubricating or protective layer.

This series, regressing to the generalized function of mucus-production, is not in any sense proposed as a genealogy of nematocysts; but at least it demonstrates that they need not have sprung, fully fashioned, Minerva-like, armed cap-à-pie, from the ectoderm (or endoderm) of the *Urcnidarien*, without precedent or example. A brilliant designer, given the basic property of a swelling substance, and given time, might be expected to produce such an object. The argument from design is unfashionable in biology, because it is supposed to place the process of elaboration beyond the range of analysis. This is only necessarily so if the designing agent is conceived as external. If the designing tendency is accepted as inherent and immanent, a property of the system, we can analyse such a system as readily as any other.

The nematocyst is remarkable enough as a mechanism; but its quality is that of a plant spore-discharge mechanism and no more. It is a finished, elaborated, 'dead' product, and for that very reason we may hope for a complete analysis of its functioning in the near future. The completion of that analysis may well afford satisfaction when it comes; but it should not be forgotten that the problem of analysis in this instance, by virtue of the 'dead' character of the mechanism, is trivial, as compared with, for example, the problems of the development and evolution of the nematocyst.

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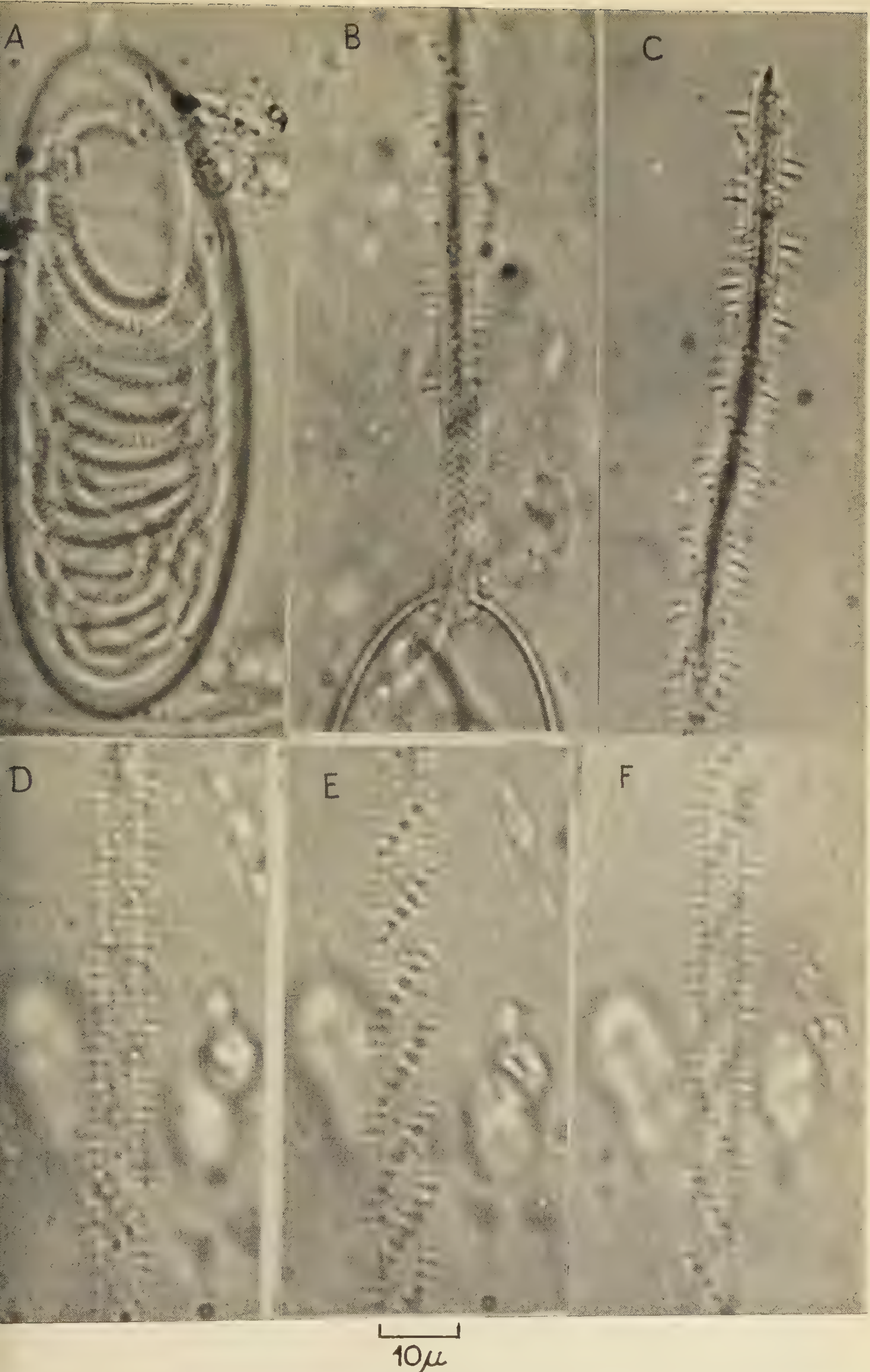

FIG. 1

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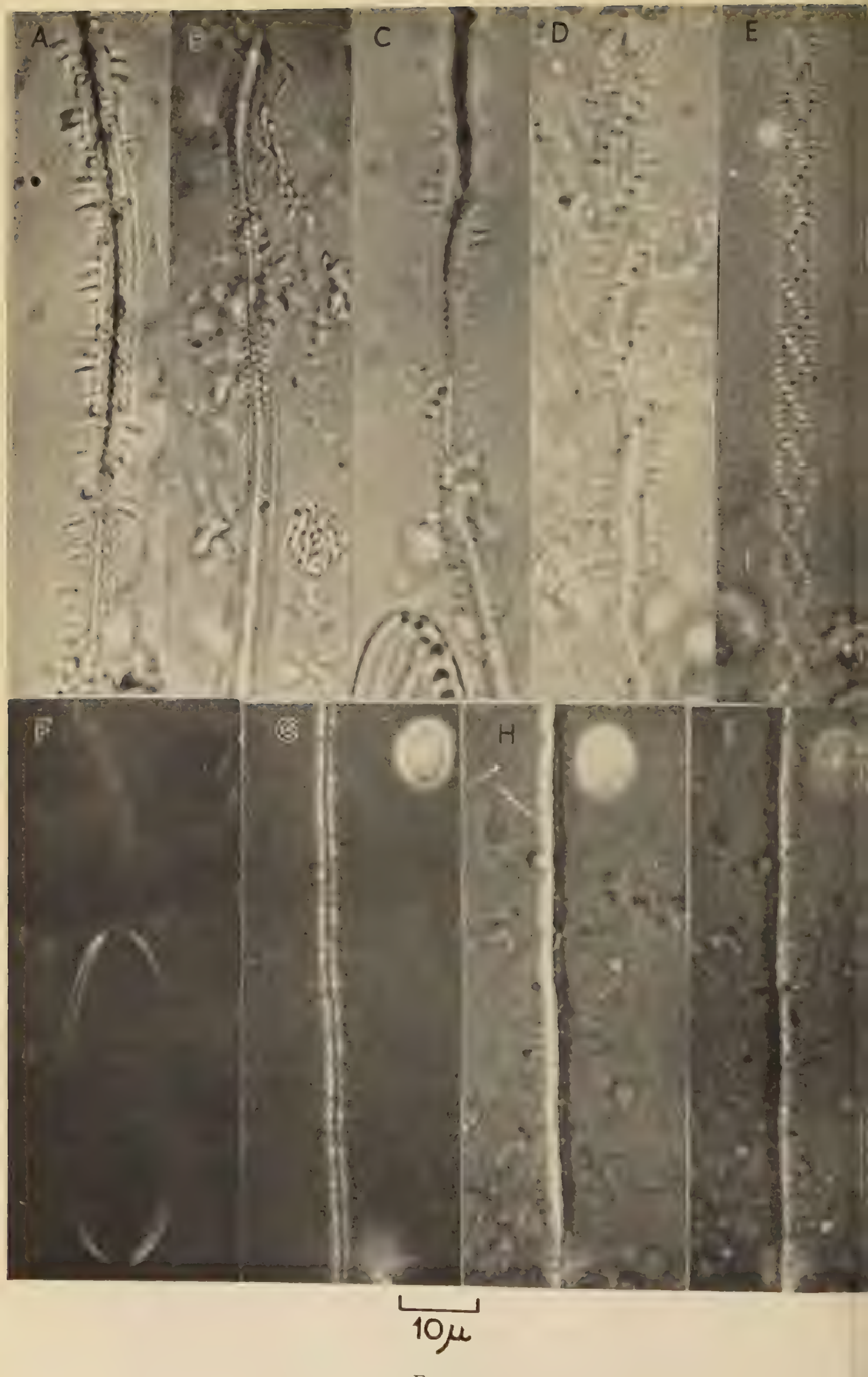

FIG. 2

L. E. R. PICKEN

Nematocysts of *Corynactis*: The Activity of the Filament during Discharge

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With two plates (figs. 1 and 2)

SUMMARY

1. The appearance of one type of nematocyst from *Corynactis* before, during, and after discharge is described. Discharge is a progressive eversion of the intracapsular filament.

2. Weill's observation that the speed at which dried nematocysts can be made to discharge depends on the quantity of water available is confirmed.

3. In water, dry isolated fragments of undischarged filament undergo changes in size which normally accompany discharge, while remaining uneverted. This reaction is an anisometric swelling of the shaft wall due to water absorption, and is largely responsible for the normal progression of discharge in nematocysts.

4. Electron microscopy has been used to investigate the morphology of various nematocysts.

INTRODUCTION

ALTHOUGH the study of nematocysts began nearly a century and a half ago, no single theory of the mechanism of discharge has been generally accepted. On the whole, recent investigators have attributed importance to the properties of the capsule wall and the fluid contents, rather than to those of the filament. The present observations and experiments relate to the nematocyst thread itself.

For this purpose, material was obtained from living *Corynactis viridis* (Allmann), supplied by the Marine Laboratory at Plymouth. Stephenson (1920–2) places this small Zoanthactiniarian with the genera *Corallimorphus* and *Isocorallion* in the family Corallimorphidae, a group which, as he emphasizes, possesses many characters common to the Madreporaria although it forms no skeleton; hence it cannot be included in the Actiniaria. Duerden (1898) and others had pointed out the Madreporarian affinities of *Corynactis* and its allies before, but without definitely separating them from the Actiniaria. Carlgren (1940) places such forms in a third group Corallimorpharia, leaving open the possibility of raising its status to that of an order parallel with the Actiniaria and Madreporaria.

A characteristic of the Madreporaria is that some nematocysts are very much larger than any found in the Actiniaria. This feature is also present in *Corynactis*, where the holotrichous isorhizas are extremely large. At the same time, these nematocysts possess a clear and characteristic barb-pattern which

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greatly assists observation, and they have therefore been chosen as material for examination. Two other types of nematocyst, and spirocysts, are also present, but these will not be considered in detail.

Normal sequence of discharge

After discharge, a holotrichous isorhiza of *Corynactis* appears as a transparent ellipsoidal capsule (up to $95 \times 35\ \mu$, Weill (1934)), from one pole of which a fairly stout, and slightly tapered shaft of relatively great length (1·5–5·0 mm.) arises, bearing three clockwise spirals of barbs. The three spirals are equidistant from one another, and if the shaft did not taper, they would be parallel (fig. 1, A). (The word 'spiral' is used throughout in its generally accepted sense, as for instance in the term 'spiral staircase'.) The barbs stand out at right angles to the surface, and are absent only from the base of the shaft and from its distal extremity. At high magnification the shaft is seen to be a tubular continuation of the capsule wall, so that it is essentially a hollow filament (fig. 1, A).

Before discharge, the filament is seen to be regularly coiled within the capsule (fig. 1, B), and in many instances the region of attachment to one pole is visible. Barbs arranged in a close hexagonal pattern (see fig. 1, C, *c*) extend almost to this junction; but since they point inwards towards the axis of the shaft, the latter is inverted compared with its condition after discharge. An operculum, which would be situated at the mouth of the capsule, has not been seen at any stage (see fig. 1, A and B).

Observation of nematocysts whose discharge has not been completed, or has been made to proceed slowly in the manner described below, confirms Doyère's original suggestion (1842) that the filament becomes everted as it shoots out from the capsule. Evagination begins at the point where the shaft

Fig. 1. Holotrichous isorhizas of *Corynactis*; light microscope photographs.

A, the capsule and base of shaft after discharge. Fresh smear in distilled water.

B, nematocyst before discharge. Dried smear in distilled water.

C, the shaft of an incompletely discharged nematocyst, showing the tip. Dried smear in distilled water.

- (*a*) Focus on the upper surface to show barb-spirals of discharged tube, and barbs forming spearhead.
- (*b*) Slightly lower plane, some barb-bases of the uneverted filament just in focus.
- (*c*) Close hexagonal pattern of barb-bases in the uneverted filament.
- (*d*) Plane of focus showing closely packed barbs of the undischarged filament pointing towards the centre of the tube.

D, a partly discharged shaft, arrested by drying, was cut through at points opposite the arrows, and flooded with distilled water. The uneverted segments of thread have elongated but the everted portions show no change. The capsule lies towards the bottom of the page.

E, cut region of shaft in an experiment similar to that shown in D. The dry, arrested shaft was cut through opposite the arrow. *X* marks the normally everted tube, *Y* the elongated, uneverted tube. Note identical spacing of the barbs in each portion.

- (*a*) This plane of focus shows regular bulging of the expanded, uneverted tube-wall.
- (*b*) Spiral arrangement of barbs in expanded, uneverted tube.

F, irregular swelling of uneverted filament seen in some nematocysts from starved *Corynactis*. A dry, undischarged capsule was ruptured, and distilled water added. Part of the thread then emerged as shown.

joins the capsule wall (see fig. 1, B), and the undischarged thread may be seen to leave the capsule, as it is drawn up the everted tube by the advancing tip. Thus the intracapsular thread uncoils like a hank of rope being paid out; and, in some cases at least, the whole of the coil of undischarged thread can be seen to rotate within the capsule as well.

The tip of the shaft is formed by a constantly renewed spearhead of apposed barbs. These flick out sharply and take their positions in each of the spiral rows. Viewed towards the tip, the three spiral rows run round the shaft in a clockwise direction. At the tip, the uneverted part of the thread can be seen to rotate in an anti-clockwise direction during discharge.

Some of these features are brought out by fig. 1, C, which shows the distal end of an incompletely discharged nematocyst at different planes of focus.

Under conditions in which the speed of discharge is reduced, the advancing tip describes a helix, and the discharged tube itself may be thrown into a permanent helix.

Observations on dried material

In order to study the mechanism of discharge, isolated nematocysts were obtained by taking smears of the oral region of a *Corynactis*. This technique, first described by Weill (1926), yields both undischarged and discharged capsules; and such preparations can be stored indefinitely without losing their properties (Weill (1934) records $5\frac{1}{2}$ years), if kept over dry calcium chloride in a desiccator. The nematocysts are easily recognizable when examined in air, in spite of poor resolution of detail and some wrinkling of the capsules. Their original appearance, however, is completely restored by the addition of water (e.g. fig. 1, B).

The advantage of such preparations lies in the fact that, as described by Weill, dry nematocysts will discharge when moistened, and the speed at which they do so depends on the quantity of water available. Dry, unexploded nematocysts of *Corynactis* discharge rapidly on the addition of distilled water, and slowly if moisture is supplied from the breath or from the air in a damp pipette, although the length of time between exposure to water and the moment when discharge begins cannot be predicted. When moist air is supplied, a discharging nematocyst is usually surrounded by a film of water, and as this evaporates, discharge comes to a standstill. The system can be set in motion again by readmitting water, unless drying has caused a permanent entanglement of the filament-coils, so that they are unable to leave the capsule freely.

The discharge of one nematocyst into air was observed to slow down and come to a stop long before eversion was complete, although the capsule itself lay within a drop of distilled water. In order to discover which part of the structure required access to water for discharge to proceed to completion, the smear was allowed to dry off so that the whole nematocyst was now in air. In making an unsuccessful attempt to deliver small volumes of water to different regions by means of a microcapillary tube, it was found that this type of instrument could be used to cut through the stationary filament, since when dry, both filament and capsule adhered slightly to the slide. The nematocyst shaft was cut through in several places, each fragment therefore consisting of an everted portion of thread surrounding an uneverted tube of the same length. A drop of distilled water was placed on the specimen, and the inner tube of each fragment was now observed to have become very much longer than the everted portion, although it was still uneverted. This observation was made before placing a coverslip on the preparation, so that mechanical pressure could not have contributed to the result.

Examination under high power showed that, unlike the condition in a normal, uneverted thread, the barbs within these elongated, uneverted segments were in spirals. The orientation of the spirals resembled that in the normally everted portions of filament (see fig. 1, E).

This experiment has been repeated many times on nematocysts whose discharge has been arrested at various stages by desiccation. By means of a fine needle with a bent tip, made either from soft glass or tungsten wire, and controlled by a Chambers' micromanipulator, any part of a dry nematocyst may be cut through.

The result of an experiment similar to that just described is shown in fig. 1, D. The addition of water to the dry specimen has caused no apparent change in the everted portion, whereas the uneverted region has clearly increased in length.

When a dry, undischarged capsule is cut in half, the pieces of filament often appear to untwist about their own axes when water is placed on the specimen. They emerge from the capsule and may assume any position. If the fragments are of appreciable length, their movements are often impeded, particularly when the cut ends cannot rotate freely, and the untwisting process may then throw the filament into tangled loops.

A change in the pattern of the barbs of the uneverted filament always accompanies its increase in size. The close hexagonal pattern of the barbs in the normal, undischarged filament is seen in fig. 1, C, *c*, and the appearance of such an uneverted shaft after elongation in fig. 1, E, *a* and *b*. These last show that after the penetration of water, the diameter of the uneverted portion equals that of the normally everted shaft, and that in both cases the pitch of the spirals is the same and the barbs are spaced identically. (The barbs themselves have not altered in size.) A characteristic feature of the elongated filament is the regular bulging of the tube wall seen in fig. 1, E, *a*. Although inspection does not reveal a spiral arrangement of the barbs in the normal, undischarged filament, an axial rotation of the shaft must occur before the coordinates of the pattern in fig. 1, C, *c* can transform to those in fig. 1, E. This rotation has been observed when undischarged fragments are exposed to water.

Such evidence shows that the uneverted filament of a holotrichous isorhiza can undergo changes which normally accompany discharge, while remaining uneverted. Since these dimensional changes depend only upon the direct influence of water on the uneverted filament, they must be caused by an intrinsic reaction of the nematocyst shaft to water absorption.

Further experiments on the swelling reaction

The action of water on the undischarged nematocyst thread causes a transformation in which a considerable amount of mechanical energy is released. It is possible that this results simply from anisometric swelling, analogous to that of wool-fibres, for example, in water. In all cases of anisometric swelling the submicroscopic structure and orientation of the material is important, and it is reasonable to suppose that in the nematocyst thread, as in other systems, chain-molecules and micelles are arranged in such a manner that water can be taken up into the system of intermicellar spaces and so give rise to the observed dilatation. While it is simplest to suppose that the material of the thread reacts immediately with water, the possibility that the reaction is mediated by an enzyme or enzymes cannot be ruled out on *a priori* grounds.

With this in mind, dry smears of *Corynactis* were heated for 5 days in an oven at 110° C. It was found that the subsequent addition of distilled water to a smear resulted in the discharge of many unexploded capsules. Since it is extremely unlikely that an enzyme would remain functional after such treatment, it seems improbable that the elongation and dilatation of the undischarged nematocyst filament should involve an enzymatic reaction. The energy of discharge therefore in all probability derives from simple swelling of the nematocyst substance.

In considering the nature of the swelling reaction, it is of interest to record an incidental observation. At one stage, cutting experiments were performed on dry nematocysts obtained from extremely small, regressed *Corynactis* which had not been fed for about 6 months. The material was then flooded with distilled water in the normal way. In several instances, such fragments of uneverted filament only swelled locally: regions in which little or no swelling appeared to have taken place alternated with others that were fully dilated, as, for instance, in fig. 1, F. This irregular swelling has not been seen in nematocysts from vigorous *Corynactis* recently obtained from Plymouth.

Swelling depends very much upon the availability of water molecules in the surrounding medium. Thus the slow discharge of dry nematocysts in 1 M sucrose is accelerated by the addition of distilled water. In 5 M sucrose (90 per cent. saturated at 10° C.) at room temperature no discharges occur, but if the solution is heated, many slow evaginations take place, gradually stopping as the medium cools. Similarly, cut fragments of uneverted nematocyst filament elongate (irreversibly, so far as can be determined) in hot 5 M sucrose, although the cold medium has no effect.

In olive oil (of approximately the same viscosity as 5 M sucrose), whether hot or cold, no discharges occur.

Electron microscopy

Various points in the morphology of discharged nematocysts of *Corynactis* have been clarified by electron microscopy. Formvar grids, covered with a dry ilm of dilute saliva, were placed on the tentacles of a *Corynactis* in air for some minutes, and in this way a certain number of discharged nematocysts were collected. The grids were then examined unshadowed, after washing with distilled water and drying.

This method of examination confirms the helical arrangement of the barbs seen under the light microscope (see fig. 1, A and C), and reveals their individual outlines (fig. 2, A). Each barb is inserted separately on an undifferentiated portion of the shaft wall, which appears to be of uniform texture. The arrested tip of a discharging shaft is seen in fig. 2, B, and the double nature of the filament at this stage in fig. 2, C (compare these with fig. 1, C). The capsule wall (fig. 2, D) is single-layered, and in the dried condition conspicuously thin (cf. fig. 1, B).

This technique has also been used to examine other types of nematocysts. The 'bottlebrush' type (microbasic mastigophore) found in *Corynactis* is shown in fig. 2, E and F, which illustrate portions of two different specimens.

Observations have been extended to the nematocycts of *Hydra* (*Chlorohydra*) *viridissima* Pallas. The proximal part of a discharged penetrant is shown in fig. 2, G. The three large barbs at the base of the filament, and also the collar region of the capsule, are seen more clearly in fig. 2, H. Such specimens were obtained by macerating a *Hydra* in dilute hydrochloric or oxalic acid on a Formvar grid, and then drying and washing with distilled water.

Discussion

In one type of nematocyst from *Corynactis*, it has been shown that isolated portions of uneverted thread lengthen by swelling when exposed to water. The close hexagonal pattern of the barbs is transformed to an open, helical arrangement, which, except that the thread is still uneverted, is identical with that in a normally discharged shaft. In the discharging nematocyst, the uneverted filament only comes into immediate contact with external water at the advancing tip of the shaft, where in fact swelling occurs. Since discharge will not proceed unless the advancing tip is supplied with water, swelling at this point must be responsible for the progressive eversion of the thread in isolated nematocysts. Continuity between the wall of the shaft and that of the capsule

FIG. 2. Siemens electron micrographs, taken at 70 kV. unless otherwise stated. The specimens were dry, and therefore collapsed. A–F, *Corynactis*; G–H, *Hydra viridissima*.

A, shaft of a fully discharged holotrichous isorhiza; capsule towards bottom of page. Note the separate insertion of barbs on undifferentiated tube-wall.

B, tip of an incompletely discharged holotrichous isorhiza.

C, shaft of an incompletely discharged holotrichous isorhiza showing a dark core of uneverted filament within the everted tube.

D, neck of fully discharged holotrichous isorhiza capsule.

E, discharged 'bottlebrush' nematocyst (microbasic mastigophore). Neck of capsule and about half the proximal portion of the shaft, showing the spiral arrangement of long barbs.

F, discharged 'bottlebrush' nematocyst. Distal portion of the shaft, which is of relatively great length. It follows without gradation the region with long barbs shown in E.

G, capsule and base of shaft of a discharged penetrant. Note that no small barbs can be seen at the base of the shaft.

H, neck of capsule and the three large barbs in another discharged penetrant. 90 kV.

ensures that once the shaft has begun to evert, it continues to do so until the end of discharge. There is no inherent tendency for the isolated thread to evert during expansion, and without this physical continuity, extending from the base of the capsule to the distal extremity of the shaft, there would be no eversion. These features are discussed further by Dr. L. E. R. Picken in a paper published in the same number of this Journal.

These results support in principle the theory put forward by Will (1909, 1914, 1915) but not as yet generally accepted, that the energy for discharge is supplied mainly by the progressive swelling of a 'colloidal substance' on the nner surface of the intra-capsular nematocyst filament. Will thought that this colloidal substance was located in three spiral bands which ran the length of the shaft, and could be seen after vital staining. Weill (1934) and others have pointed out that several kinds of nematocyst do not possess such bands at any stage, and electron microscopy has shown that they are not present in the types of nematocyst examined here. If, however, the function of these swelling bands' is assigned to the shaft wall itself, Will's hypothesis is seen n essentials to be correct. At least it attributes the progressive elongation and dilatation of the thread, as the tip advances during discharge, to the swelling properties of the thread itself.

This work was begun at the suggestion of Dr. L. E. R. Picken, whom I wish to thank for his generous help and advice, both during the study and in the preparation of the manuscript. It was concluded while the author held a D.S.I.R. assistantship under Dr. C. F. A. Pantin, F.R.S., to whom I wish to express my sincere and very grateful thanks for his advice and continual encouragement. I am indebted to the D.S.I.R. for their financial support, to Dr. V. E. Cosslett and Mr. H. E. Pearson for facilities which enabled the electron microscope to be used, to Professor T. A. Stephenson, F.R.S., for help with the classification of *Corynactis*, and to Miss E. W. McConnachie for reading the manuscript.

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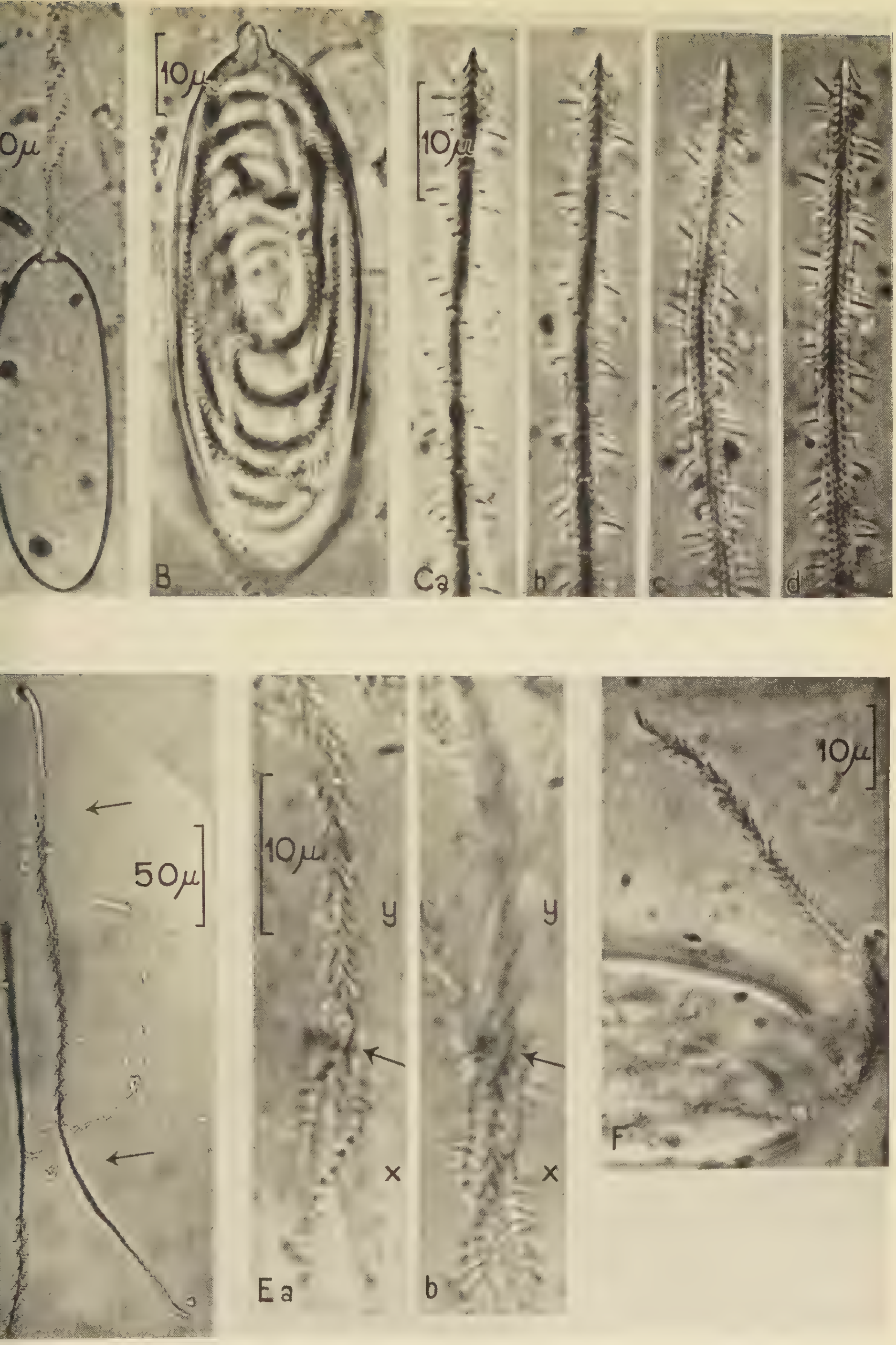

FIG. 1

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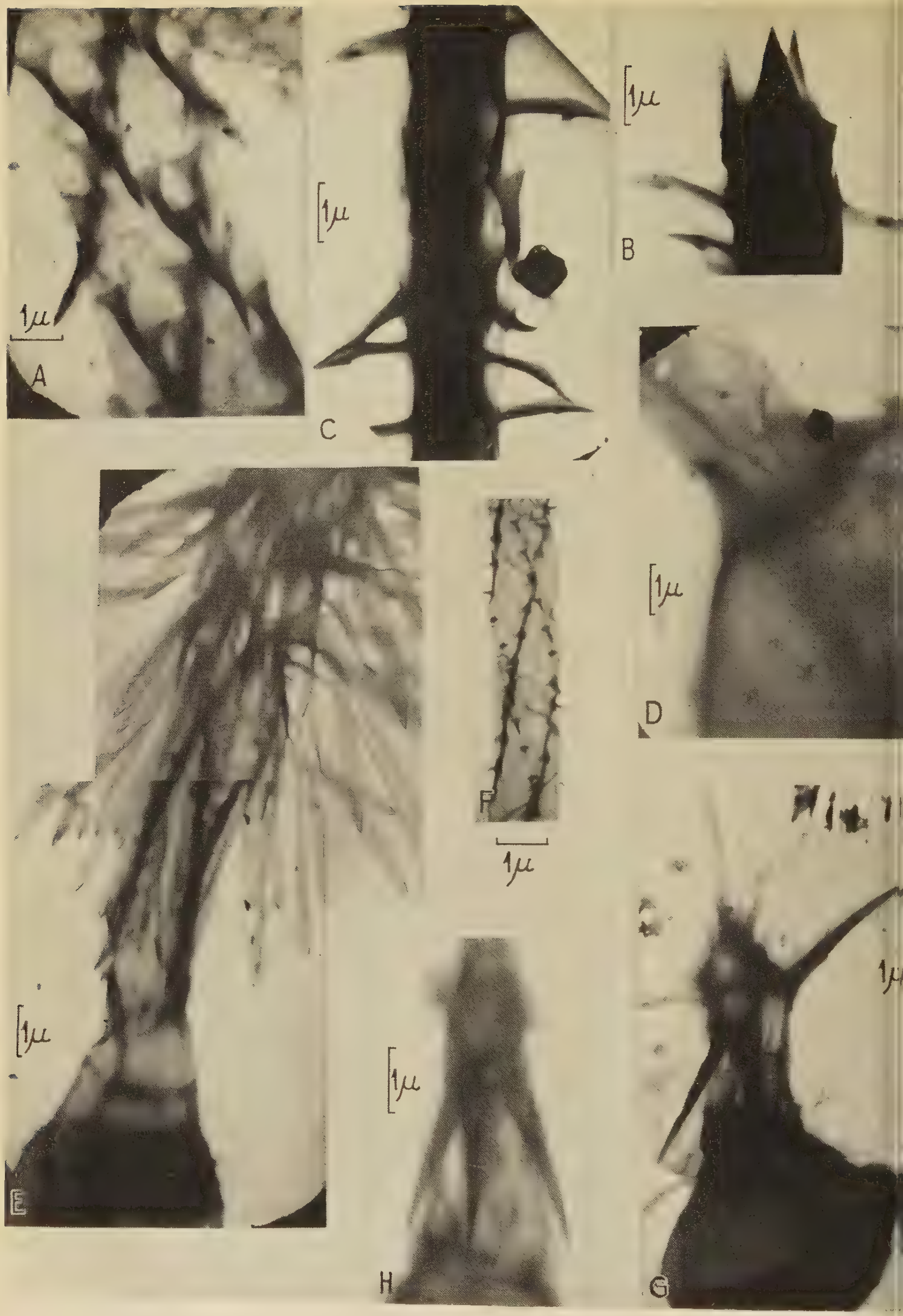

Fig. 2

E. A. ROBSON

Miscellaneous Contributions to Microtechnique

By JOHN R. BAKER AND BARBARA M. JORDAN

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SUMMARY

1. The glycerine in Mayer's glycerine and albumen serves no useful purpose.

2. If about *one-half* of the haematoxylin in such solutions as those of Heidenhain and Ehrlich be oxidized by sodium iodate, the dyes are ready for use the moment they have been made up and also keep well.

3. In using crystal violet for staining chromosomes, it is best to fix the dye by an aqueous (instead of the usual alcoholic) solution of iodine.

DURING the course of our teaching and research, we have worked out what we believe to be better methods of performing certain familiar techniques. We think that others might be glad to have particulars of them. The three techniques that will be discussed in the present paper are the attachment of sections to slides, the preparation of solutions of haematoxylin, and the staining of chromosomes with crystal violet and iodine.

Adhesive albumen for the attachment of sections to slides

For the past 70 years Mayer's glycerine and albumen has been the standard adhesive used in attaching sections to slides (Mayer, 1883). The inventor of this method mixed glycerine with an equal volume of filtered egg-white, dipped a brush into this fluid, and spread a thin layer of it over a slide. He then placed a paraffin section on it and put the slide on a water-bath. Thus the section was flattened on undiluted glycerine and albumen. The sole purpose of the glycerine, as Mayer stated clearly, was to keep the adhesive wet during this process. Finally, the albumen was precipitated by warmth and drying, and the section adhered to the glass.

Although Mayer's solution is used to this day, almost to the exclusion of other adhesives, yet it is improbable that anyone nowadays flattens sections as Mayer did. It is usual either to put 5 drops of his solution in about 10 c.c. of water and to flatten the sections on this, or else to flatten them on warm water in a basin and then transfer them to Mayer's solution smeared on a slide. In neither case does the glycerine perform the function intended by Mayer.

The apparent uselessness of the glycerine suggested a trial of albumen alone. The results were from the first perfectly satisfactory, so far as flattening and adhesion were concerned, but the solution did not keep well. Mayer himself at first used phenol (1883) and then changed to sodium salicylate at 1 per cent. (1887). The latter acts well in the presence of glycerine, but does not preserve simple albumen solutions indefinitely. We have found sodium

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p-hydroxybenzoate an admirable medium for this purpose. (If there should be any difficulty in obtaining the pure substance, 'Moldex' may be used instead. It is obtainable from Messrs. R. Campbell and Co., 7 Idol Lane, Eastcheap, London, E.C. 3.)

Our solution is prepared as follows:

To 100 c.c. of 1 per cent. (or 0·9 %) aqueous sodium chloride solution, add 0·2 gm. of sodium *p*-hydroxybenzoate. Mix this solution with an equal volume of egg-white. Stir well. Centrifuge until the supernatant fluid is clear. The supernatant fluid is the adhesive.

We call this product 'adhesive albumen' (AA). For paraffin sections we dilute the AA in the usual proportions (5 drops to about 10 c.c. of distilled water), flood the slide with this, float the sections on it, and flatten them by warming. When the excess of fluid has been drained off and the preparation dried on the hot plate, the sections adhere very firmly. Workers who attach sections after first flattening them in a basin of warm water should use AA exactly as though it were Mayer's fluid.

AA is perfectly suitable for the attachment of celloidin sections by Richardson's technique (1952).

Half-oxidized haematoxylin

It was pointed out by Unna long ago (1891) that the ordinary solutions of haematoxylin used for staining contain three constituents—unripe, ripe, and over-ripe portions of the dye, the latter forming a precipitate. In other words, the solutions contain haematoxylin, haematein, and further oxidation-products. Only the haematein is a dye. The haematoxylin keeps up the strength of the solution by gradual conversion to haematein. The further oxidation-products are useless. If haematein is used instead of haematoxylin in making up the solution, there is continual reduction in the strength of the stain with the passage of time.

Unna added sulphur to partially ripe haematoxylin solutions, to check oxidation at an early stage (presumably by the production of small quantities of sulphuretted hydrogen). He sometimes used this 'half-ripe' haematoxylin as a dye, sometimes kept it as a stock solution, fully oxidizing the required quantity from time to time with hydrogen peroxide.

Our purpose has been to produce haematoxylin solutions that would be usable the moment they had been prepared, but would nevertheless be stable. To do this we have used sodium iodate as oxidizing agent, but have allowed only about one-half of the amount that would oxidize the whole of the haematoxylin. The rest of the haematoxylin remains in the solution and is gradually oxidized to haematein by atmospheric oxygen, thus maintaining or increasing the strength of the solution.

Sodium iodate was used to oxidize haematoxylin by Mayer (1903), who allowed 0·2 gm. of the salt to 1 gm. of haematoxylin, in the belief that this amount was a little less than what was necessary to oxidize the haematoxylin fully. Actually 0·187 gm. of sodium iodate suffices to oxidize 1 gm. of haematoxylin to haematein (Hansen, 1905). In our solutions we allow 0·1 gm. of sodium iodate to 1 gm. of haematoxylin, and thus oxidize a little more than one-half of the latter.

We boil the haematoxylin with the iodate, and the roughly half-oxidized solutions thus produced can be used directly they have been made up. In a stoppered bottle they gradually increase slightly in staining power. Heidenhain's, Regaud's, Ehrlich's, and Mayer's haematoxylins, made up in half-oxidized form, were stored for 6 months and then compared with the same solutions made up on the day of the test. In all cases the fresh dye acted well, but the stored dye was slightly stronger.

We make up the *half-oxidized versions* of the various dyes as follows:

Heidenhain's haematoxylin (1896)

Haematoxylin	5 gm.	Distilled water	950 c.c.
Sodium iodate	0·5 gm.	Alcohol (96 %)	50 c.c.

Put the haematoxylin and the sodium iodate into the water. Heat till the water just boils. Cool. Add the alcohol.

We find that the alcohol may be omitted, 1,000 c.c. of water being used instead of 950 c.c. Heidenhain used the alcohol to dissolve the haematoxylin quickly, but this is not necessary when heat is used.

Regaud's haematoxylin (1910)

Haematoxylin	10 gm.	Glycerine	100 c.c.
Sodium iodate	1 gm.	Absolute (or 96 %) alcohol	100 c.c.
Distilled water	800 c.c.		

Put the haematoxylin and sodium iodate into the water. Heat till the water ust boils. Cool. Add the glycerine and alcohol. (Distilled water might presumably be substituted for the alcohol.)

Ehrlich's haematoxylin (1886)

Haematoxylin	6·7 gm.	Glycerol	333 c.c.
Sodium iodate	0·67 gm.	Absolute (or 96 %) alcohol	333 c.c.
Distilled water	333 c.c.	Acetic acid (glacial)	33 c.c.
Potassium alum	18 gm.		

Put the haematoxylin and sodium iodate into the water. Heat till the water ust boils; then remove from the flame and add the alum. Stir from time to ime. Mix together the glycerol, alcohol, and acetic acid, and stir them into he haematoxylin solution when the latter has cooled to 50° C.

The amount of alum is just sufficient to leave a small precipitate.

Mayer's acid haemalum (1903)

Haematoxylin	1 gm.	Potassium alum	50 gm.
Sodium iodate	0·1 gm.	Chloral hydrate	50 gm.
Distilled water	1,000 c.c.	Citric acid	1 gm.

Put the haematoxylin and sodium iodate into the water. Heat till the water just boils; then remove from the flame and add the alum. Stir from time to time. When the solution is cool, add the chloral hydrate and citric acid.

Dobell's haematein (1914)

Haematoxylin . . .	10 gm.
Sodium iodate . . .	1 gm.
Alcohol, 70 % . . .	1,000 c.c.

Put the constituents in a flask provided with a reflux condenser. Heat till the fluid boils. Cool. Do not detach the reflux condenser until the fluid is cool.

Hansen's 'Trioxyhaematein'

Haematoxylin .	7·1 gm.	Iron alum . .	44·4 gm.
Sodium iodate .	0·71 gm.	Ammonium sulphate .	6·2 gm.
Distilled water .	1,000 c.c.		

Put the haematoxylin and sodium iodate into the water. Heat till the water just boils; then remove from the flame, add the iron alum and ammonium sulphate, and allow to cool, stirring from time to time.

Hansen himself believed that his 'Trioxyhaematein' represented a particular stage in the oxidation of haematoxylin, beyond haematein, and he gave careful instructions for preparing it. A complicated routine, based on Hansen's instructions, is usually followed. However, our simple method of preparation gives a stain that appears to act in the same way as Hansen's. Whether the solution is made up according to Hansen's instructions or ours, it is not stable under the ordinary conditions of laboratory use, for a precipitate forms and the ability to stain falls off rather quickly. However, if a bottle is filled to the top with our solution and well stoppered, the stain will still be in good condition 6 months later, though it will require filtering.

Crystal violet and iodine for staining chromosomes

This is an invaluable technique, especially for the study of meiotic prophases. The chromosomes are precisely stained, while the cytoplasm is of glassy transparency. Very thick sections can be used.

The method was introduced by Newton (1926), who gave no detailed instructions. He stained with gentian violet (a mixture of crystal violet in unstated proportions with other dyes), and secured it in the chromosomes by adding 1 per cent. of iodine (and 1 per cent. of potassium iodide) to the dehydrating alcohols. The principle is the same as that of Gram's bacterial stain. More precise details of technique were given by Huskins (1927) and La Cour (1931). Their techniques give excellent results, but are nevertheless open to the following criticisms.

After staining with gentian violet, both Huskins and La Cour treated the sections (or smears) with iodine/potassium iodide/80 per cent. alcohol. Since iodine is soluble in 80 per cent. alcohol, the function of the potassium iodide is unexplained. In the alcoholic iodine two antagonistic processes are at work. The alcohol tends to remove the dye from the chromosomes, while the iodine tends to fix it in them. If the final result is unsatisfactory, it is difficult to judge whether to increase or decrease the period in this solution. Very little resistance to extraction by alcohol is given, for, as Huskins says, the slides must be taken 'almost as rapidly as possible' through absolute alcohol. Since dehydration is incomplete, the sections must pass through the expensive clove oil before entering xylene; and to remove the clove oil, prolonged soaking (15 min.) in three lots of xylene is advised. The first xylene becomes badly contaminated with clove oil, and is thus spoilt for use in other methods. As La Cour says of this method, 'Staining is an art which requires practice to reach perfection'.

It seemed rational to fix the crystal violet in the chromosomes by means of iodine dissolved in a medium (aqueous potassium iodide solution) that does not extract the stain. If this is done, there is no need to rush the dehydration of the sections, and the use of clove oil is unnecessary. We believe that our method is not only more rational, but also much simpler and easier than the one usually employed. We prefer pure crystal violet to the redder mixture of dyes (gentian violet).

Our technique is as follows. We usually fix in Sanfelice's fluid (1918), but sometimes in Zenker's; the latter, though generally used only as a routine fixative in histology and micro-anatomy, is quite a good fixative for some chromosomes. We embed in soft paraffin and usually cut sections at 14 μ.

Two solutions are required for staining:

Crystal violet, 1 per cent. aqueous.
Iodine, 1 per cent. in 2 per cent. aqueous potassium iodide solution.

The technique is as follows:

(1) Bring sections to water.
(2) Crystal violet, 15 minutes.
(3) Rinse in distilled water.
(4) Iodine solution, 1 minute.
(5) Rinse in distilled water.
(6) 70 per cent., 90 per cent., and first absolute alcohol, about 10 seconds in each.
(7) Second absolute alcohol, about 1 minute.
(8) Xylene, two lots.
(9) Mount in D.P.X. (Canada balsam is also suitable, but the stain sometimes fades in this medium.)

If a section is understained, this can be corrected with the next slide by lengthening stage (2) or shortening stage (7). If it is overstained, the reverse applies.

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Standardization of Methyl Green for Specific Staining of Egg-shell Material in a Trematode

By J. D. SMYTH

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SUMMARY

1. The specificity of a number of samples of methyl green for egg-shell material in *Fasciola hepatica* was investigated.

2. A method for distinguishing between specific and non-specific stains by paper chromatography is described.

3. Specific stains gave almost identical chromatograms with an Rf value of 0·83 for both green and violet constituents. Non-specific samples gave chromatograms with an Rf of 0·65 for the green constituent and an Rf of 0·84 for the violet constituent. The liquid phase used was a mixture of organic solvents in the following proportions: ethanol, 100; water, 80; *n*-butanol, 100; cellosolve, 100.

4. The application of this technique has led to the production of a methyl green, named Methyl Green M.2, specific for shell material in *Fasciola*.

INTRODUCTION

AQUEOUS methyl green has a marked affinity for the untanned egg-shell material in trematodes and pseudo-phyllidean cestodes, and under carefully controlled conditions acts as a specific stain for this material (Smyth, 1951*a*, 1951*b*).

Several workers have since reported (private communication) that the method has failed to work in their hands, in that nuclei and other organelles also stained green or blue-green by this technique. Since methyl green is notoriously inconsistent in its composition, it was suspected that the fault most likely lay in the sample of stain used. Samples of methyl green were obtained from a number of different manufacturers and their specificity for egg-shell material tested. The various samples were then investigated by paper chromatography; by this means it was possible to develop a method of standardizing a sample of methyl green with the required degree of specificity for egg-shell material.

COMPOSITION OF METHYL GREEN

Data concerning the composition of methyl green, given by Conn (1946), may be briefly summarized here. Methyl green is one of the tri-amino-tri-phenyl-methane group, which includes a number of other well-known dyes. As the seventh methyl group is very loosely attached, there is always some methyl or crystal violet impurity present. Owing to its instability,

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standardization of this stain is difficult. It is soluble in water (4·8 gm. per 100 ml.) and alcohol (0·75 gm. per 100 ml.).

TESTING SPECIFICITY OF METHYL GREEN FOR EGG-SHELL MATERIAL

Fresh specimens of *Fasciola hepatica* were fixed in 5 per cent. formol-saline and embedded in wax; 7·5 μ sections were cut. These sections were stained in 0·5 per cent. *aqueous* solutions of the different samples of methyl green, according to the procedure in the original technique (Smyth, 1951*b*). It is particularly stressed that only neutral aqueous solutions were used. The acid solutions, in which methyl green is widely used as a nuclear stain, are unsuitable.

RESULTS OF STAINING

The results of staining are summarized in Table 1. Four of the samples, obtained from two different manufacturers (A and E), stained the egg-shell material specifically. The eggs in the lower uterus (i.e. before tanning and hardening) and the shell globules in the vitelline cells and in the vitelline ducts, stained brilliant green or blue-green, the green being retained by no other tissues. The methyl violet impurity stained the nuclei violet. The remaining six samples of the stain, obtained from four manufacturers (A, B, C, D), were all non-specific in their staining reactions, acting mainly as nuclear stains but staining other organelles also.

CHROMATOGRAPHIC ANALYSIS

About 10 c.c. of saturated aqueous solutions of the various samples of methyl green were prepared and stored in a dark cupboard.

Battery jars were used as vessels and standard paper chromatography procedure (ascending method) was adopted. Small circular spots (2 μl.) of the dyes were placed on the starting-line of the paper with the aid of a fine pipette—one spot to each 3 inches of paper-width. Whatman No. 1 paper was used throughout. The chromatograms were allowed to run for a convenient time (about 24 hours); they were then removed and dried, and the frontier of each spot measured. A number of solvents and mixtures of solvents were tried as the liquid phase; the following mixture was found to give clear spots and was used throughout: ethanol 60 c.c.; *n*-butanol, 100 c.c.; cellosolve, 100 c.c.; water, 80 c.c.

RESULTS OF CHROMATOGRAPHIC ANALYSIS

The results are shown in Table 1. As is usual in chromatographic work, provided that the temperature was fairly constant and fresh solvents were used, the Rf values obtained were remarkably consistent. The figures quoted in table 1 are averages of at least three results, the experimental error being not greater than ±0·02.

These figures revealed that the samples of methyl green investigated fell sharply into two groups giving very typical chromatograms; these are shown diagramatically in fig. 1. Type I, containing all the samples that were specific for egg-shell material, showed a green and a violet spot occupying almost identical positions in the chromatogram. This overlap of the green and violet spots produced a bluish spot with a violet front edge and a green back edge, indicating that the Rf of the methyl violet is slightly higher than that of the methyl green.

TABLE 1. *Rf values for the green and violet ions separated from different samples of methyl green*

Temperature 18°–20° C. (For composition of liquid phase, see text)

Stain No.	*Specific for egg-shell material*	*Rf values*	
		Green spot	*Violet spot*
A 1	yes	0·84	none
A 2	yes	0·82	0·84
A 3	yes	0·83	0·83
E	yes	0·84	none
A 4	no	0·68	0·85
B	no	0·62	0·85
C 1	no	0·69	0·87
C 2	no	0·65	0·85
D 1	no	0·63	0·82
D 2	no	0·64	0·82

Type II, containing samples which stain non-specifically for egg-shell material, showed a characteristic methyl violet spot at about 0·84 (mean), as n the previous type, and a very elongated green spot with a mean Rf of 0·65 (range 0·62–0·69). The exact borders of this latter spot were often difficult to define, and in some cases it almost extended up to and bordered on the methyl violet spot.

DISCUSSION

Uncertainty regarding the composition of a stain is often a source of error n cytology, and most workers are familiar with stains that bear the same name but give widely different results. Typical examples are the basic fuchsin in the Feulgen reaction and the various sudans in the fat demonstration and ssociated techniques. The results obtained by paper chromatography suggest that this technique has many advantages in certain cases for testing such stains, and in the case of methyl green has proved particularly effective.

It has been shown that in the chromatogram of the specific stain, the green ppears as a small spot. This result suggests that a fairly pure green substance s present. Moreover, since its Rf value is almost identical with that of methyl violet, it is likely that this stain is made up of almost pure methyl green, as the chemical composition of these two substances is very similar.

On the other hand, in the chromatograms of the non-specific stains, the green constituent appears as a long green streak—a result which indicates the presence of a number of green compounds, which may include some pure

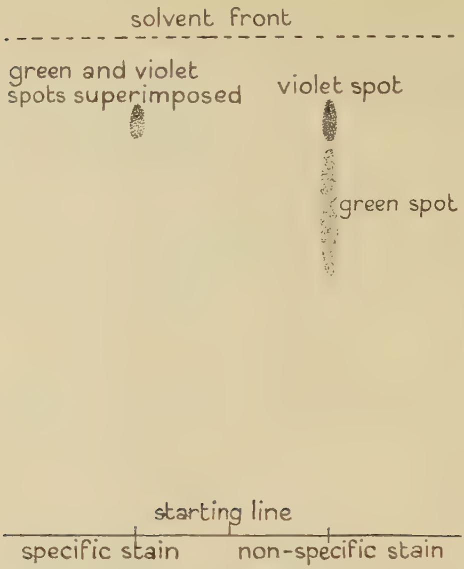

FIG. 1. Typical chromatograms obtained with samples of methyl green, specific and non-specific for egg-shell material in *Fasciola hepatica.*

methyl green. It is possible that this streak represents a number of breakdown products of methyl green.

From these results it was possible to conclude that in order to stain egg-shell material specifically in *Fasciola* (and possibly in trematodes in general) a methyl green with an Rf value of 0·83 in the stated solvent must be used.

Thanks to the co-operation of Messrs. G. T. Gurr, a methyl green with this characteristic has been produced: it has been named Methyl Green M.2.

I am indebted to the Medical Research Council of Ireland for financial aid in support of this work.

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Studies upon the Gram Reaction of the Basiphil Cells of the Anterior Pituitary

Part II. Observations upon the Effects of Various Methods of Fixation

By R. R. WILSON AND C. L. FOSTER

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With one plate (fig. 1)

SUMMARY

To investigate the effects of fixation on the Gram reaction in the pituitary basiphil granules, frozen sections were made from fixed and unfixed glands, paraffin sections from frozen-dried glands, and paraffin sections in the usual way from whole glands, as well as from several pieces of glands embedded in one block. The fixatives were various well-known mixtures and single substances. Staphylococci were also studied, after treatment with some of the fixatives.

The basiphil granules were always Gram-positive after fixation in formaldehyde-saline, formaldehyde-Zenker, Zenker's fluid, and Zenker stock-solution. Frozen sections of unfixed glands, and sections of frozen-dried glands, were always Gram-negative; but such sections were usually Gram-positive if left in formaldehyde-saline for at least 12 hours before staining. All other fixatives studied gave negative results. The staphylococci were always Gram-positive.

These findings, and some questions arising from them, are briefly discussed.

INTRODUCTION

IN a recent communication (Foster and Wilson, 1952) we reported the first results of a study of the Gram-positive properties of the cells of the human anterior pituitary. It was found that the Gram-positive reaction occurred regularly in the cytoplasmic granules of the basiphil cells, but never in those of the acidophils, nor in the cytoplasm of the chromophobes. The Gram-positive reaction in the basiphils was not abolished by ribonuclease, though it was abolished by hot oxygenated bile salt, after the method of Henry and Stacey (1946). Moreover, the distribution of ribonucleic acid, as shown by pyroninophilia and ribonuclease extraction, did not correspond with the distribution of Gram-positive staining. For these and similar reasons we concluded that it was unlikely, or at least unproven, that ribonucleic acid was the essential factor in determining the Gram-positive reaction of the basiphil granules.

These results were obtained from human post-mortem material, which was fixed in 10 per cent. formaldehyde-saline, embedded in paraffin, and sectioned

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in the usual way. We pointed out that this method differs from that of most workers studying the Gram reaction in bacteria, whose material is fixed by heat. We decided accordingly to investigate the effects of various methods of fixation. The purpose of the present paper is to record the results of this work.

MATERIALS

Human pituitaries obtained at post-mortem have again been used; specimens fixed more than 12 hours after death have been rejected, those fixed less than 6 hours after death have been specially sought. We have also used pituitaries from the rabbit, rat, and monkey (macaque); and we made a few observations on staphylococci.

METHODS

As our principal object was to study the effects of various fixatives on the Gram reaction, it was necessary—

(i) to exclude as far as possible the effects of reagents used in embedding and sectioning, which was done by making frozen sections of fixed and unfixed glands, and in two cases (rabbit) by the freezing-drying technique;

(ii) to keep embedding and cutting processes, and the actual Gram staining, as constant as possible, which was done by embedding a number of specimens in a single paraffin block.

The details of the methods now follow:

Human pituitaries were either halved horizontally or cut into more numerous pieces by antero-posterior and transverse sections.

(*a*) Halved pituitaries fixed in formaldehyde-saline were sectioned on the freezing microtome, the sections received in distilled water, mounted on albumenized slides, and stained.

(*b*) Halved unfixed glands were treated in the same way.

(*c*) Halved unfixed pituitaries were sectioned on the freezing microtome, the sections received in formaldehyde-saline and kept in it for varying periods, mounted on albumenized slides, and stained.

(*d*) Two rabbit pituitaries were embedded in paraffin by the freezing-drying technique, the sections cut in the usual way (some being kept in formaldehyde-saline for varying periods), mounted and stained without denaturation in alcohol.

(*e*) Halved human pituitaries, or whole animal pituitaries, were fixed in various fixatives, embedded singly in paraffin, sectioned and stained as usual.

(*f*) Pituitaries were cut with a razor-blade into pieces, up to ten in number. The pieces of each gland were then fixed separately in different fixatives, and after 12–24 hours were taken side by side through the various stages of paraffin infiltration and finally embedded in a single block of wax. Sections vere cut from this so that all the pieces were represented on the same lide.

(*g*) A few 24-hour cultures of staphylococci were obtained. Suspensions of ach culture in water were prepared. Ten films were made of the suspension f each culture, in the ten compartments formed on a slide by grease-pencil ines; the films were allowed to dry in the air. Each of the ten films was then overed by a drop of one of ten fixing reagents (formaldehyde-saline and the ine 'single substances' listed below), which were left in position for 5 minutes o overnight, with precautions against drying; the fixatives were finally washed ff with water. Films of each suspension were also fixed by heat.

(*h*) The fixing reagents used fall into two groups: (i) mixtures, (ii) single ubstances.

(i) The mixtures were: 10 per cent. neutral formaldehyde-saline (included among 'mixtures' because Crawford and Barer (1952) have shown that the saline has its own effect). Zenker's fluid. (The 'Zenker' made up in the Biology Department contains no sodium sulphate; we have not observed any difference in the results.) 'Zenker stock' (Zenker's fluid without acetic acid). Formaldehyde-Zenker or Helly's fluid (the same as Zenker's fluid except that 40 per cent. formaldehyde is substituted for the glacial acetic acid). Potassium dichromate acetic acid (3 per cent. potassium dichromate 95 ml., glacial acetic acid 5 ml.). Mercuric chloride acetic acid (saturated aqueous mercuric chloride 95 ml., glacial acetic acid 5 ml.). Bouin's fluid. 'Susa'.

(ii) The single substances were acetaldehyde, 13 per cent. (to give a molecular concentration roughly equivalent to that in formaldehyde-saline); benzaldehyde (pure); ethyl alcohol, 80 per cent.; potassium dichromate, 3 per cent.; mercuric chloride, saturated aqueous solution; acetic acid, 10 per cent.; osmium tetroxide, 2 per cent.; chromic acid, 1 per cent. The solvent or diluent was distilled water in all cases.

(*i*) The stains used were: Gram's stain; periodic acid/Schiff; and acid uchsin/aniline blue/orange G. Details of composition and technique were as iven in our last paper (1952).

(*j*) Appropriate controls were used throughout. If, say, Gram's stain was eing used on a section of Susa-fixed rabbit pituitary, a section of formaldeyde-fixed rabbit pituitary was stained side by side with it. When a number f pieces were embedded together in one block, one of them was always ormaldehyde-fixed and was regarded as a control for the Gram staining. Vhen a section from such a block was stained by Gram's method, the next ections in the series were stained by the periodic acid/Schiff method, or by cid fuchsin/aniline blue/orange G, or both, to act as a control for the presence f basiphil cells.

(*k*) On suspicion that results were influenced by the thickness of the ections, sections were cut from a formaldehyde-fixed rabbit gland at 5, 10, 5, and 25 μ, and stained by Gram's method as usual.

RESULTS

First, it should be made clear that our study has been concentrated on the granules of the basiphils. Other structures (such as nuclei, collagen, and elastin) may also be Gram-positive. We have noticed this in our material, but the ensuing reports refer only to the basiphil granules.

The results may be briefly summarized by saying that the Gram reaction was positive only in preparations treated with formaldehyde-saline, formaldehyde-Zenker, Zenker's fluid, and Zenker stock. (See table 1.) This was constant when the glands were initially fixed in these fluids. A positive result was usual, but not invariable, when frozen sections of unfixed glands, or sections of frozen-dried glands, were left in formaldehyde-saline for at least 12 hours. Frozen sections of unfixed glands, and sections of frozen-dried glands stained without further treatment, were always Gram-negative.

TABLE 1. *Results of Gram reaction in basiphil granules of pituitary, after various fixatives*

Fixatives	*Gram reaction*
(i) *Mixtures*	
10 % formaldehyde-saline	+
Zenker's fluid	+
Zenker stock	+
formaldehyde-Zenker	+
potassium dichromate acetic acid	—
mercuric chloride acetic acid	—
Bouin's fluid	—
'Susa'	—
(ii) *Single substances*	
13 % acetaldehyde	—
benzaldehyde	—
3 % potassium dichromate	—
sat. aqueous mercuric chloride	—
sat. aqueous picric acid	—
10 % acetic acid	—
2 % osmium tetroxide	—
1 % chromic acid	—

FIG. 1 (plate). A, low-power view of a section of four pieces of a human pituitary, separately fixed but embedded, cut, and stained together. Gram's method (no counterstain). The letters indicate the fixatives used (*f* = formaldehyde-saline, *z* = Zenker's fluid, *b* = Bouin's fluid, *a* = 80 per cent. alcohol). The small rectangles in the *f* and *a* sections indicate the positions of B and C.

B shows the typical appearance of the basiphil cells stained by Gram's method after fixation in formaldehyde-saline. Only basiphil granules are stained. (From the place shown in A, *f*; for comparison with C.)

C shows the appearances observed with staining by Gram's method after fixation in 80 per cent. alcohol. Nuclei and colloid are stained, but no granules. (From the place shown in A, *a*; for comparison with B.)

From this we conclude that the fixation, and not subsequent processes, is responsible for the results of staining by Gram's method.

Negative results were obtained with material fixed in potassium dichromate/acetic acid, mercuric chloride/acetic acid, and all the other mixtures and single substances mentioned above.

The staphylococci were Gram-positive in all cases. The conclusion here is that the Gram reaction in these organisms is different from that seen in the pituitary, which is perhaps not surprising.

Differences in the thickness of sections were not found to affect the Gram reaction. This test was prompted by the negative Gram reaction in the unfixed frozen sections: we suspected at first that this might have been due to the greater thickness of the frozen sections compared with the paraffin ones.

The results with the controls—see 'Methods (*j*)'—were not altogether clear-cut. When a section of a formaldehyde-fixed gland was used as a control for, say, a section of a Zenker-fixed gland, then the control was always Gram-positive. When a formaldehyde-fixed piece was embedded as a control along with pieces fixed in other reagents, then the formaldehyde-fixed piece was always Gram-positive. But, when neighbouring sections in the series cut from such blocks were stained with periodic acid/Schiff or acid fuchsin/aniline blue/orange G, as a control for the presence of basiphils, the results in the pieces fixed in single substances were so unsatisfactory that we could not be sure whether or not basiphil cells were in fact present. This doubt applied particularly to the small pieces embedded together. Formaldehyde-saline always gave positive results with periodic acid/Schiff.

We consider, however, that we are justified in reporting the negative results given above, for the following reasons. First, the results were negative in whole glands, and in glands that were halved or quartered. Secondly, the reports are based on two or more observations (except for mercuric chloride/acetic acid). Thirdly, the human glands were from elderly subjects, and basiphil cells were very numerous in them.

The cytoplasmic appearances in preparations reported as Gram-negative were not identical in all cases. In formaldehyde-fixed specimens the Gram-negative cells were invariably of a clear yellow colour. In contrast to this, in preparations fixed by potassium dichromate/acetic acid, and to some extent in those fixed by chromic acid, many cells showed a brownish or purplish coloration which at first sight suggested a rather poor Gram-positive result. The coloration however was not only much weaker than the true Gram stain, but also it was diffuse and not restricted to the granules; therefore we cannot regard it as a typical positive result.

DISCUSSION

It seems to us that the salient findings are these: that, on the one hand, positive results are got with formaldehyde-saline, with formaldehyde-Zenker, Zenker's fluid, and Zenker stock, while, on the other hand, negative results follow fixation in acetaldehyde and benzaldehyde, in potassium dichromate alone and potassium dichromate/acetic acid, in chromic acid, in mercuric chloride alone and mercuric chloride-acetic acid, and in Bouin's fluid.

The positive Gram reaction after formaldehyde, contrasted with the negative results after acetaldehyde and benzaldehyde, may be regarded as yet another instance of a property of formaldehyde not shared by some other aldehydes.

Examination of the other results seems to show that Gram staining in the basiphil granules of the pituitary depends upon fixation being done in either formaldehyde alone or mercuric chloride and potassium dichromate together. This conclusion in turn depends upon the assumption (based on appearances) that the Gram-positive results obtained by these two methods are in fact identical. We feel justified in making this working assumption, keeping in mind that it may later be proved erroneous.

The next problem appears to be to find a reaction or reactions on cell components, for example proteins, that is likely to be shared by formaldehyde and mercuric chloride plus potassium dichromate (see French and Edsall, 1945). We hesitate to do more than state this problem; the more so because formaldehyde and potassium dichromate are not protein precipitants, while mercuric chloride is (Baker, 1945). We wonder if perhaps in this combination the potassium dichromate effect predominates over or prevents the effect of the mercuric chloride; if that occurred, the mercuric chloride might act on the potassium dichromate-fixed material in such a way as to produce a Gram-positive result, which is not given by either substance alone.

A similar problem is presented by the constantly positive results with formaldehyde, in contrast to the negative results with Bouin's fluid (which of course contains formaldehyde).

The effect of formaldehyde on unfixed frozen sections and frozen-dried sections also interests us. The Gram stain was negative unless the formaldehyde was allowed to act for a number of hours, although the reagent must have penetrated quickly to all parts of these very 'thin slices'. This suggests a chemical reaction. Crawford and Barer (1951) found that morphological changes in cells continue for many hours during fixation by formaldehyde.

The results obtained in our work suggest that authors who describe staining-methods should be careful to specify exactly the fixatives they use.

We acknowledge with thanks the interest shown by Professor W. D. Newcomb, of the Department of Morbid Anatomy; and the assistance of Miss Margaret Wilson, technician in the Department of Biology.

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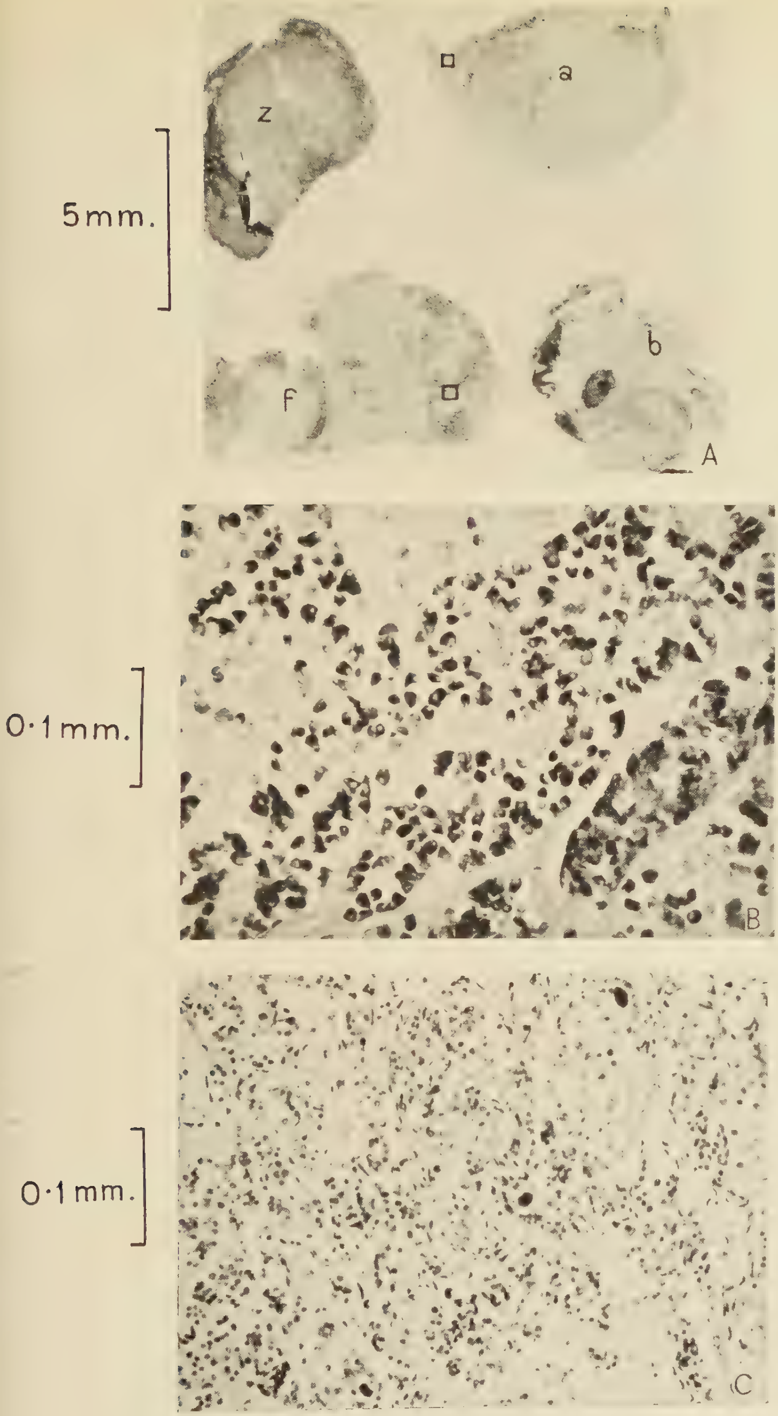

FIG. 1—R. R. WILSON and C. L. FOSTER

The Localization of Dehydrogenase Activity and Sulphydryl Groups in Wool and Hair Follicles by the use of Tetrazolium Salts

By G. E. ROGERS

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With four plates (figs. 2–5)

SUMMARY

Three tetrazolium salts are used as histochemical indicators of dehydrogenase activity in the skin of sheep and other mammals. Of these compounds 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) is shown to be the most satisfactory. It enables various histological structures of the wool follicle to be conveniently demonstrated.

In the presence of appropriate substrates and DPN and ATP, the intracellular deposition of the granular formazan in frozen sections has revealed the distribution of succinic, α-glycerophosphoric, lactic, and malic dehydrogenases, and regions where some phosphorylated carbohydrate intermediates promote reduction. Metabolic activity in the Malpighian layers and in the undifferentiated epidermal cells of the hair matrix reduces INT enzymatically up to the pre-keratinous zone. Here the protein-bound sulphydryl groups reduce INT and produce intense red staining which is easily distinguished from the granular enzymic staining.

The distribution of cytochrome oxidase in the wool follicle resembles the distribution of dehydrogenase activity.

INT is used for the estimation of succinic dehydrogenase in skin and kidney tissues.

INTRODUCTION

MANY active processes associated with the growth, development, and maintenance of the epidermis, follicles, and hairs have been detected through the application of histological and biochemical methods. The histology of the hair follicle and the distribution of certain enzymes, nucleic acids, and other constituents have been studied (Giroud and Bulliard, 1930; Auber, 1950; Giroud and Leblond, 1951; Leblond, 1951; Hardy, 1952), but there is comparatively little information on the glycolytic and oxidative metabolism of skin and particularly on the localization of the enzyme systems concerned in these processes. Several workers have studied carbohydrate metabolism in skin. Thus Barron, Meyer, and Miller (1948) studied respiration and glycolysis in surviving rat skin, and Medawar (1947) and Bullough (1952) investigated the association of carbohydrate metabolism with epidermal mitosis. It is known, for example, that inhibitors of glucose utilization prevent epidermal cell division, and that intermediates of the Krebs cycle increase O_2 uptake by the cells and accelerate mitosis.

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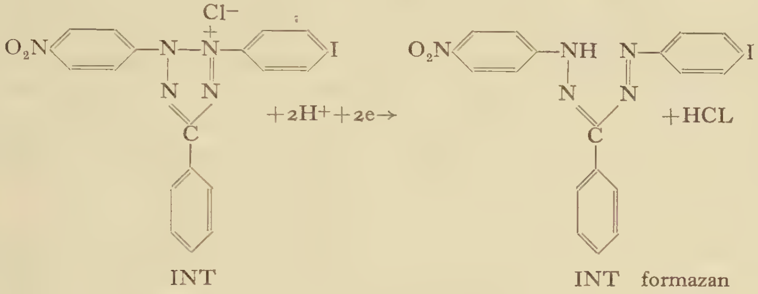
Such studies, however, have not been extended to the active epidermal cells of hair follicles although some evidence has been obtained by Ellis, Gillespie, and Lindley (1950) that dehydrogenase activity and Krebs cycle intermediates exist in the follicles of sheepskin. In view of the active cellular growth and synthesis of keratin which occurs in the wool follicle, an investigation has been made in this laboratory of the distribution of enzyme systems concerned with carbohydrate metabolism in sheepskin. In this work tetrazolium salts have been used as indicators of dehydrogenase activity since there is abundant evidence of the efficacy of these substances as histochemical reagents for detecting dehydrogenases in tissues (Seligman and Rutenberg, 1951; Smith, 1951; Neumann, 1952; Shelton and Schneider, 1952). Several methods have been published for the quantitative estimation of dehydrogenase activity in cells and cell particle suspensions (Black and Kleiner, 1949; Nordmann, Nordmann, and Gauchery, 1951; Fahmy and Walsh, 1952), but although several isolated enzyme systems are also capable of reducing tetrazolium salts (Mattson, Jensen, and Dutcher, 1947; Brodie and Gots, 1951; Kun, 1951), the pathways by which tetrazolium salts accept electrons are not clear. Several tetrazolium derivatives have been synthesized for histochemical use, since the original 2,3,5-triphenyl tetrazolium chloride (TTC) was found to be histologically unsatisfactory (Shelton and Schneider, 1952). They include blue-tetrazolium (BT) which is 3,3′-dianisole-bis 4,4′(3,4-diphenyl) tetrazolium chloride (Rutenberg, Gofstein, and Seligman, 1950), neotetrazolium (NT) (Antopol, Glaubach, and Goldman, 1950), and tetrazolium-violet (TV), the latter two being derivatives of BT (Shelton and Schneider, 1952). Rutenberg, Gofstein, and Seligman (1950) used BT for the estimation of several dehydrogenases in kidney and other tissues and also for the histological localization of succinic dehydrogenase (Seligman and Rutenberg, 1951). More recently several other enzymes of the Krebs tricarboxylic acid cycle have been localized in kidney frozen sections by the use of NT and BT with the addition of various co-factors to the substrate (Farber, Sternberg, and Dunlap, 1952). Furthermore, Shelton and Schneider (1952) surveyed the use of several tetrazolium salts as histochemical reagents for dehydrogenases and concluded that NT was the most satisfactory, histologically and enzymatically. However, apart from the application of one derivative (TTC) (Steigleider, 1952), they have not been previously used in histochemical investigations of skin.

In experiments to be described, skin blocks, slices, and frozen sections are shown to reduce tetrazolium salts readily, and by the application of these compounds specific dehydrogenase activity is localized in hair follicles and epidermis. The iodo-nitro-derivative, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT), is used in the present study in preference to other tetrazolium derivatives because there is considerably less diffusion of the intracellular deposit of granular formazan from its site of formation than with the other compounds. Moreover, a larger number of substrates react with it, and uniform staining and excellent delineation of histological structures are obtained in frozen sections. The only previous reference to its application in the study of animal tissues is that of Calcutt (1952), who mentioned its use for staining muscle but gave few details. Seligman, Gofstein, and Rutenberg (1949) synthesized the 3-iodo-phenyl derivative incorporating radio-iodine and used it for studying the reducing activity in the tissues of mice after injection, and Atkinson, Melvin, and Fox (1950) prepared several iodo-derivatives, including INT, and noted the more rapid reduction of INT in plant tissue when compared with other derivatives. INT has also been used for the quantitative determination of corticosteroids (Henly, 1952).

INT is a pale yellow substance, soluble in water. In its reduced form (INT formazan) it is reddish purple (maximum absorption at 465 mμ in solution in acetone), and insoluble in water. The reduction of INT occurs thus:



MATERIALS AND METHODS

Source and preparation of skin

Foetal or adult sheepskins, usually of a fine cross-bred type, were obtained from the abattoirs immediately after slaughter and stored in a moist atmosphere at 6° C. Fresh samples of skin from the mouse, rat, guinea-pig, rabbit, and man have also been used. Although most of the experiments were carried out within 24 hours, no significant changes in reactivity were observable during 4 days' storage.

Skin blocks, slices, or frozen sections were used. With sheepskin the wool was shorn as closely as possible and frozen sections, 50 μ to 80 μ thick and 1 cm. long, were cut perpendicular to the epidermal surface with a Spencer sledge-type microtome and transferred to buffer-Ringer (2 vol. 0·05 M phosphate buffer at pH 7·4+1 vol. Ringer) at room temperature for up to 2 hours until used. The skin blocks (4 mm. × 4 mm.) or slices (1 mm. thick) were cut with a razor blade and immersed in buffer-Ringer.

Experimental conditions

For carrying out the histochemical reactions the tetrazolium reagents (TTC and INT, Pal Chemicals Ltd.; BT, Dajac Labs.) were prepared by mixing 1 ml. substrate solution (usually 0·2 M at pH 7·4) and 1 ml. 0·5 per cent. tetrazolium salt and diluting to 5 ml. with buffer-Ringer. When additional compounds were added in 1 ml. amounts, the buffer-Ringer solution was reduced accordingly.

Test tubes containing the solutions and skin specimens were incubated at 37° C. for times varying from 2 minutes to 2 hours and the specimens were then washed in buffer-Ringer. Sections were mounted with glycerogel for microscopic examination.

RESULTS

Observations with TTC

Reactions in the presence of various substrates

In preliminary experiments sheepskin blocks, incubated with 0·1 per cent. TTC, were found to contain a heavy deposit of the red formazan within 2 hours in the absence of added substrate, owing to the presence of sufficient endogenous substrates and co-factors in the skin. However, the rate of reduction decreased with decreasing thickness of the skin slice and no reduction occurred in frozen sections, even after the addition of substrates such as glucose or succinate, together with DPN (1 mg./5 ml.) and ATP (1 mg./5 ml.). Although it has been noted by other workers in experiments with other tissues (for example, Shelton and Schneider, 1952), the reason for the inactivity of frozen sections towards TTC is not known. Freezing alone was not responsible, as skin blocks and slices were still active if frozen with CO_2 even for much longer periods.

The substrates tested for their effects on the reduction of TTC by skin included amino-acids and various intermediates of carbohydrate metabolism (see Table 1). The extent of their effect could only be assessed semi-quantitatively by visual observation of the intensity of staining of the tissue, since a quantitative method could not be adapted to non-uniformly stained blocks of skin. The following substrates caused increased reduction of TTC: cysteine, glutathione (GSH), ascorbic acid, succinate, lactate, and glucose. It is likely that cysteine, GSH, and ascorbic acid activated the enzyme systems which utilized endogenous metabolites. Inhibitors of cysteine desulphurase, such as 0·001 M semicarbazide and 0·001 M hydroxylamine, did not prevent the reaction. The reduction of TTC is enzymic since it did not occur in skin which had been subjected to buffer-Ringer at 90° C. for 5 minutes. Cysteine and GSH are unable to reduce TTC at pH values less than 9·0. Furthermore, reduction in the presence of succinate was strongly inhibited by 0·005 M malonate, which suggests the presence of succinic dehydrogenase (see Table 2).

Of a number of known metabolic inhibitors tested, only four were found to inhibit the reduction of TTC by skin in the presence of succinate. These were malonate (0·02 M), *o*-iodosobenzoate (0·04 M), N-ethylmaleimide (0·04 M), and potassium cyanide (0·005 M). Heavy metals were also inhibitory but 0·01 M iodoacetate, 0·004 M *o*-aminophenylarsonate, 0·004 M sodium azide, and 0·01 M urethane were without effect. It is possible that the inhibitors are not taken up by the cells as readily as the substrate.

Tests in Thunberg tubes under anaerobic conditions showed no acceleration in reduction of TTC in skin blocks or frozen sections, nor could the reduction of TTC by succinate be induced in frozen sections, by the simultaneous presence of cysteine to activate the enzyme systems.

An exact pattern of dehydrogenase distribution in the follicle could not be obtained with TTC, owing to the formation of large needle-shaped crystals, which continued to grow at the expense of smaller crystals and granules during fixation and embedding. Such crystals frequently protruded from one follicle layer into another. Furthermore, non-uniform staining was obtained owing to the concentration gradient set up during diffusion of the TTC into the skin. The results obtained with TTC were in accord with, but less well defined than, those obtained with INT.

All ages of sheepskin, from early foetal to adult, stained in the epidermal layers. In foetal skin approximately 35 days old before follicle buds had begun to form, the germinating layer of the embryonic epidermis was well defined by the deposition of formazan. The growth and development of the wool follicles could also be followed when samples of skin from embryos of ages advancing from 35 days to 100 days old were examined in this manner. The approximate foetal ages were estimated by reference to Cloete (1939) and Carter (1943).

Observations with BT

A sample of BT was tested at 0·1 per cent. concentration on frozen sections of sheepskin in the presence of added succinate and DPN-ATP. After 2 hours a very slight but patchy reduction in the usual zones had occurred. Anaerobic conditions had no effect on the rate of reduction. Blocks of skin and skin slices readily reduced the reagent, but examination by frozen sections showed the deposit to be non-uniform. Of several substrates tested with BT on frozen sections only ascorbic acid in the presence of DPN and ATP yielded granular formazan deposits. These occurred in the zones localized by INT and light purple staining appeared in the zones known to contain sulphydryl groups. The nature of the reduction in the presence of ascorbic acid is not clear and requires further study.

The use of TTC and BT was finally discontinued in favour of INT which gave much more satisfactory results.

Observations with INT

Frozen sections

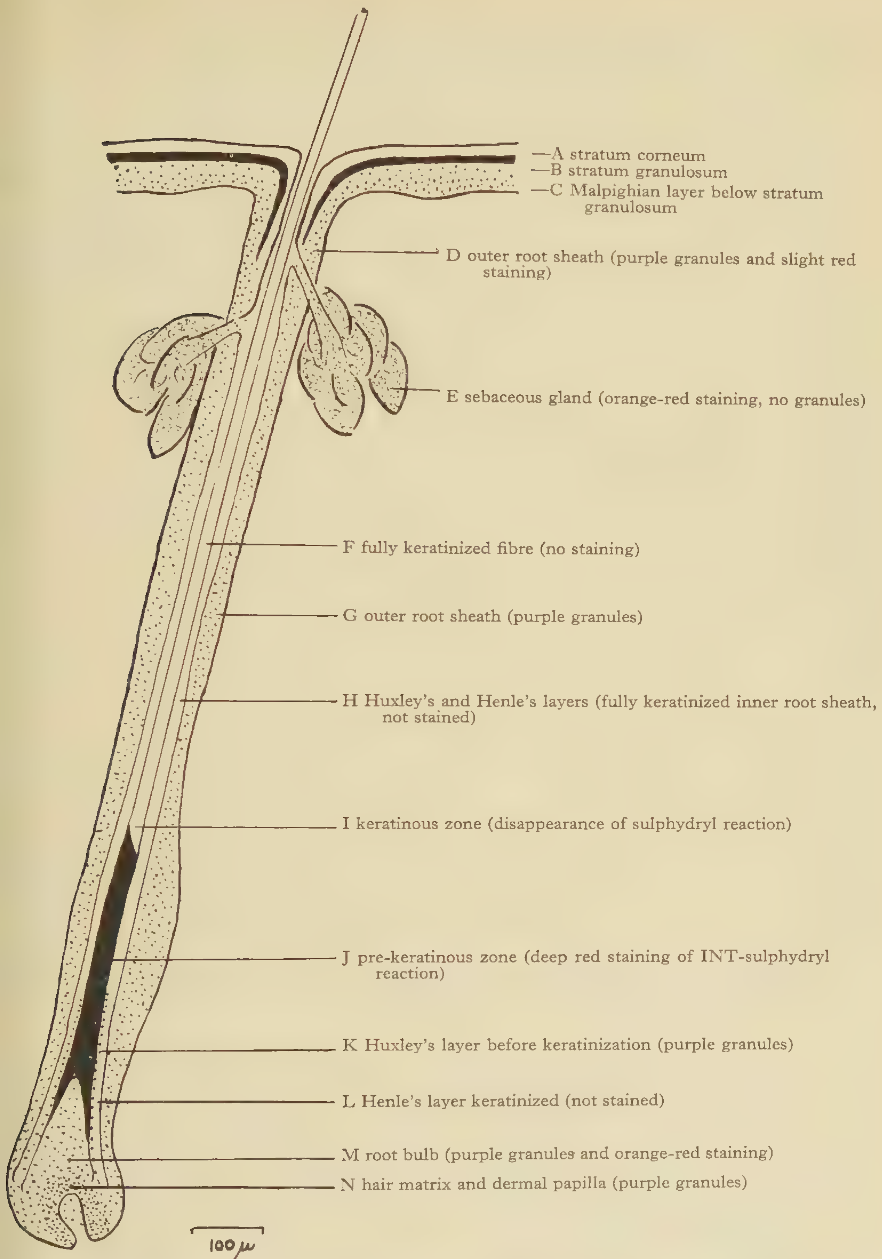
When skin blocks were incubated in the presence of 0·1 per cent. INT, the purplish granular formazan deposit produced remained finely dispersed for the experimental period and the beginning of reduction was recognizable within 5–10 minutes instead of requiring 30–60 minutes as with TTC. Of greater importance was the observation that *frozen sections* reduce INT, and this occurs even in the absence of added substrate. After 10 minutes' incubation, microscopic examination showed the purplish formazan granules to be very lightly distributed in the actively metabolizing areas of the follicles fig. 5, B). After 30 minutes, staining was sufficiently intense for the gross structure of the skin and follicles to be evident from inspection of the frozen sections. It was found that staining in the absence of added substrate could be kept to a minimum by using sections about 50 μ to 80 μ thick. Samples of tissue in this form were entirely satisfactory for testing the effect of various added substrates and localizing the specific dehydrogenases concerned. Such conditions were not always readily attained with the other tetrazolium salts (Seligman and Rutenberg, 1951).

INT-formazan distribution in sheepskin without added substrate

Microscopic examination of frozen sections mounted in glycerogel, after 30 minutes' incubation with INT alone, revealed areas of metabolic activity (fig. 1). The Malpighian layers were stained, the intensity of staining diminishing as the cells approached the upper strata of the epidermis and being absent when keratinization was complete. In the follicle, intracellular deposits of purple granules were easily observable in the outer root sheath and in Huxley's layer, before keratinization had occurred. Henle's layer was not stained as it is keratinized before leaving the bulb, and both Huxley's and Henle's layers when keratinized were sharply distinguished from the reactive outer root sheath. The sebaceous glands evidently have intense activity as they were stained orange red, as previously described for TTC. The undifferentiated cells of the bulb were stained and the highest activity was evident in this region, but this gradually diminished as the cells approached the zone of the cortex just above the constriction of the bulb. However, at this level a dark red reaction developed and extended through the proximal third of the follicle. This region gives the well-known nitroprusside reaction and the recently developed Prussian blue stain (Chèvremont and Frederic, 1943) for protein-bound sulphydryl groups, the so-called pre-keratinous zone (figs. 1; 2, A, B, and C). The same reaction, apparently due to sulphydryl groups, was detected also in the *stratum granulosum* at the stage of keratinization below the *stratum corneum* of the epidermis (figs. 1; 2, C; 5, C and D). This non-enzymic staining was uniformly deep red after 5 minutes' incubation of the frozen section in INT, and consequently developed some time before the dehydrogenase reduction was observed. Although the reaction product was red and non-granular, small crystals of formazan formed in this zone when stained sections were held for one week.

Increased enzymic reduction of INT in the presence of added substrate

The addition of certain substrates to the incubation medium promoted the reduction of INT in frozen sections of sheepskin. The most active was succinate, which produced dense purple staining after 10 minutes' incubation. Under these experimental conditions there was very little staining in the absence of substrate. Both DPN and ATP (1 mg./5 ml.) were added to maintain the enzyme systems. The DPN–ATP addition increased the control staining also, but this still remained minimal and did not interfere with the detection of added substrates. As shown in Table 1, the following substrates were effective at 0·04 M concentration after 10 minutes' incubation:


—A stratum corneum
—B stratum granulosum
—C Malpighian layer below stratum granulosum
D outer root sheath (purple granules and slight red staining)
E sebaceous gland (orange-red staining, no granules)
F fully keratinized fibre (no staining)
G outer root sheath (purple granules)
H Huxley's and Henle's layers (fully keratinized inner root sheath, not stained)
I keratinous zone (disappearance of sulphydryl reaction)
J pre-keratinous zone (deep red staining of INT-sulphydryl reaction)
K Huxley's layer before keratinization (purple granules)
L Henle's layer keratinized (not stained)
M root bulb (purple granules and orange-red staining)
N hair matrix and dermal papilla (purple granules)
100 µ

IG. 1. Diagrammatic representation of the distribution of succinic dehydrogenase and sulphy-
yl groups in a wool follicle, revealed by the INT method. The colours in the epidermis are
follows: stratum corneum, not stained; stratum granulosum, light red sulphydryl staining;
alpighian layer below stratum granulosum, purple formazan granules from enzymic activity.

Succinate, α-glycerophosphate, lactate, and malate (dehydrogenases), fumar ate (fumarase and malic dehydrogenase), fructose-1 : 6-diphosphate, glucose-1 phosphate, and 3-phosphoglycerate (glycolysis intermediates).

TABLE 1. *Activation of tetrazolium reduction by skin in the presence of variou substrates*

Substrate 0·04 *M* (*Na salts*)	*INT* (*DPN–ATP*) *Sheepskin frozen sections*	*TTC Sheepskin block and slices*
Glucose	—	+
Succinate	+	+
Lactate	+	+
α-Glycerophosphate	+	
Malate	+	
Fumarate	+	
Fructose-1 : 6-diphosphate	+	
Glucose-1-phosphate (K salt)	+	
3-Phosphoglycerate	+	
Cysteine	reduced directly	+
Gluthathione		+
Ascorbic acid		+
Oxaloacetate	—	—
α-Ketoglutarate	—	—
Pyruvate	—	—
Citrate	—	—
Aconitate	—	—
Xanthate (saturated at pH 7·4)	—	—
Glutamate	—	—
Proline	—	—
Tyrosine (saturated at pH 7·4)	—	—
Aliphatic amino acids	—	—
Control	—	—

Incubation time: INT 10 minutes; TTC 60 minutes. Temperature 37° C Control—zero reduction. Intensity of staining is not indicated.

Neither cysteine, GSH, nor ascorbic acid could be tested since they readily reduce INT at room temperature and pH 7·4.

INT-formazan distribution in sheepskin in the presence of added substrate

(*a*) *Succinate*. Sections removed from the INT-buffer-succinate solution at 2- to 15-minute intervals during incubation revealed a close parallel between

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FIG. 2. (plate). A, adult sheepskin, frozen section incubated 5 minutes in INT-succinate Succinic dehydrogenase activity is localized in the outer root sheath and particularly in the hai matrix of the bulb. The sulphydryl reaction is also evident. Note the dense staining in the sebaceous glands.

B, similar to A but at higher magnification. The formazan can be seen lightly deposited in the outer root sheath and localized in the hair matrix of the bulb around the dermal papilla. The intense INT-sulphydryl reaction is visible in the pre-keratinous zone.

C, adult sheepskin, frozen section in INT-succinate. This shows the general distribution of succinic dehydrogenase activity throughout the wool follicle (cf. fig. 1), but not all the reactive zones can be observed.

he succinic dehydrogenase staining and that due to sulphydryl groups (figs. ; 2, A, B, and C). Thereafter the enzymic formazan intensity began to increase o much that cell detail was partially obscured.

The cells of the Malpighian layers, particularly the outer root sheath, were tained purple (figs. 2, C; 3, B and C) and formed a reactive zone which was bserved as a continuous layer along the skin surface and the length of the ollicle terminating in the stained bulb (figs. 2, C and 3, A). Above the level of he sebaceous glands, which were deeply stained orange-red (fig. 2, A), the urple granules in the outer root sheath were associated with light orange-red taining, evidently owing to partial solution by lipide from the sebaceous lands. Similar staining was found in the Malpighian layer of the epidermis ist above the purple-stained basal layer (*stratum germinativum*). This is gain probably due to lipide, which is known to occur in soft keratin, but the ulphydryl reaction in this layer also contributed. Examination of the intensely tained bulb showed a tendency for concentration of formazan in the vicinity f the hair matrix (figs. 2, B and 3, A). In the remainder of the bulb towards he constriction, the purple deposit was also distributed against a background f orange-red staining. This may have been partly due to lipide but the staining was close to the red zone of sulphydryl reactivity. Huxley's layer was eadily observed before keratinization (fig. 3, B), but Henle's layer and the nner root sheath cuticle were not stained since they are keratinized at a much ower level (fig. 3, B and C).

The red reaction due to sulphydryl groups, developed at or just above the ulb constriction, was continuous along the proximal third of the follicle, radually fading and finally disappearing at the keratinization level (figs. 1 and , C). When fibres in the sections were cut transversely at this level, the reacion was intensified at the cut ends, evidently owing to increased diffusion. 'his effect was also noted to a slight degree with the nitroprusside reaction. None of the layers of the inner root sheath gave any sulphydryl reaction igs. 3, C and 4, D), but such reactivity of the scales in the pre-keratinous reion was readily observable in sections stained after fibres had been pulled out, eaving a group of scales behind. Evidently the binding of the scales to the

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FIG. 3 (plate). A, high-power view of succinic dehydrogenase activity in the wool root bulb. ote the granules in the undifferentiated region of cell division (matrix), and little or no tivity in the dermal papilla. (INT-succinate.)

B, high-power view of succinic dehydrogenase activity at the level of Huxley's layer before eratinization (see fig. 1). INT-formazan has been deposited in the outer root sheath, Huxy's layer, and the sulphydryl reactive pre-keratinous zone. Henle's layer (keratinized) is not ained.

C, same as B but in the keratinous zone (region of disappearance of sulphydryl reaction; see . 1). Huxley's layer is now keratinized and does not stain. The inner root sheath and cuticle not show any sulphydryl staining at any stage. The decrease in the intensity of the INTlphydryl reaction in this region can be seen. Succinic dehydrogenase activity remains in the iter root sheath.

D, adult sheepskin, paraffin section treated with 0·1 per cent. INT at pH 7·4. The lphydryl zone in the pre-keratinous region is stained, and the reaction has also occurred other structures such as the outer root sheath.

cortical layer is not established at this stage (figs. 4, C and D). The wool roots, removed by plucking, displayed succinic dehydrogenase activity in the active bulb cells attached to the fibre and the sulphydryl reaction in the prekeratinous zone (fig. 4, A and B). The dehydrogenase activity was inhibited by malonate.

Other skin structures in which this enzyme was perceptible were the erector-pili muscle, sweat glands, blood-vessels, and occasional cells scattered throughout the dermis.

(*b*) *α-glycerophosphate, lactate, malate, and fumarate.* Staining in the presence of these substrates was similar in distribution to that observed with succinate. With α-glycerophosphate the bulb staining tended to be less intense than with succinate. The staining with lactate and malate was less intense than with α-glycerophosphate, and fumarate was even less active, as expected from the necessity for its prior conversion to malate. More pronounced staining in the bulb than in the remainder of the follicle was observed in the presence of malate and lactate, whereas the converse was found with fumarate.

(*c*) *Fructose-1:6-diphosphate, glucose-1-phosphate, 3-phosphoglycerate.* The distribution of formazan in the follicles and epidermis produced by these phosphorylated intermediates was markedly different from that observed with other substrates. It was characterized by non-uniform staining with intense purplish-black deposits in the bulb and in scattered regions of the outer root sheath, particularly proximal to the bulb. The sebaceous glands were also stained. Although the intensity of staining was interesting, its non-uniform distribution probably indicated non-uniform cell permeability to these compounds. The impermeability of many cells to such compounds is well recognized.

INT-formazan distribution in other tissues

The efficacy of INT for detecting dehydrogenases was demonstrated also for muscle and kidney. Frozen sections of sheep kidney cortex, 35–50 μ thick, were extremely active in reducing INT in the presence of succinate (fig. 5, E). The reduction was inhibited by malonate, and control sections without added substrates were scarcely stained. After 2 minutes' incubation, purple formazan granules were deposited in the renal tubules but not in the glomeruli. The

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FIG. 4 (plate). A, sulphydryl reaction by application of nitroprusside to isolated wool roots.

B, sulphydryl reaction and slight enzyme activity in isolated wool roots obtained with INT-succinate.

C, adult sheepskin. Frozen section prepared after wool fibres had been removed from th skin. Incubated in INT-succinate. Enzyme staining is present in the outer root sheath. The scale structure remaining possesses sulphydryl groups and is densely stained. The unreactive inner root sheath cuticle is discernible.

D, as in C, but the section was treated previously with 5 per cent. formol-saline. The enzym staining is absent but the sulphydryl reaction in the scale structure remains.

E, adult sheepskin, frozen section, incubated for 10 minutes in INT-succinate. Succini dehydrogenase and sulphydryl activity are apparent.

F, as in E, but after pre-treatment with 5 per cent. formol-saline. The enzyme activity has bee destroyed but the sulphydryl reaction remains.

picture did not change up to 10 minutes, but subsequently cell detail became obscure. Furthermore, in contrast with other tetrazolium salts, the kidney sections were uniformly and intensely stained in every instance.

In all cases the appearance of stained sections mounted with glycerogel did not change during 6 weeks except for growth of the granules into small crystals.

Factors influencing enzymic-INT reduction

Both endogenous and substrate-activated enzymic-reduction of INT by skin was destroyed by immersion of frozen sections for 5 minutes in 5 per cent. formol-saline (fig. 4, E and F) or buffer-Ringer at 90° C. Various inhibitors of carbohydrate metabolism were tested but only malonate (figs. 4, E; 5, A and B), heavy metals, and sulphydryl-coupling compounds had a significant effect (see Table 2). Anaerobic conditions in Thunberg tubes failed to increase the rate of reduction of INT.

TABLE 2. *Effect of various factors on enzymic tetrazolium reduction*

Addition or treatment	*Final Concentration (M)*	*Substrate and hydrogen acceptor*		
		INT+succinate	*INT+glucose-1-phosphate*	*TTC+succinate*
Malonate . .	0·02	Inhibition	No effect	Inhibition
KCN . . .	0·005	No effect	,,	Slight inhibition
NaF . . .	0·004	,,	,,	No effect
NaN_3 . .	0·004	,,	,,	,,
Urethane . .	0·01	,,	,,	,,
Iodoacetate . .	0·01	Inhibition	. .	No effect
p-iodosobenzoate .	0·04	,,	. .	Inhibition
N-ethylmaleimide	0·04	,,	Inhibition	,,
5 per cent. formol-saline . .	(pre-treatment)	,,	,,	,,
Heat: 90° C. for 5 mins. . .	(pre-treatment)	,,	,,	,,
Heavy metals, e.g. Cu^{++} . .	. .	,,	. .	,,
Anaerobiosis .	. .	No effect	No effect	No effect

0·1 per cent. INT and TTC, 10 minutes' and 60 minutes' incubation at 37° C. respectively. Concentration of substrates, 0·04 M.

FIG. 5 (plate). A, as in 4, E, but showing inhibition of succinic dehydrogenase, in the presence of malonate and succinate, by much less deposition of formazan.

B, control section, without malonate or succinate. Only slight enzymic deposition of formazan observed, but the sulphydryl zone is stained.

C, mouse skin, frozen section, INT-succinate. Distribution of dehydrogenase activity and sulphydryl groups is similar to that found in sheepskin.

D, human skin, frozen section, INT-succinate. Dehydrogenase activity and the INT-sulphydryl reaction occur in the Malpighian layer.

E, kidney cortex, frozen section incubated in INT-succinate for 5 minutes. The deposition of INT-formazan granules can be readily seen in the tubules but not in the glomeruli.

Reduction of INT by sulphydryl groups in sheepskin sections

This reaction is of particular interest since, unlike the nitroprusside and Prussian blue reactions, it may be satisfactorily applied to frozen sections under conditions which do not affect the histological structures and it is useful for comparing the distribution of dehydrogenases in relation to the pre-keratinous zones (figs. 4, E and F; 5, A and B). That the reduction is due to thiol groups is suggested not only by the histological distribution but also by the ready reduction of INT at pH 7·4 by a range of thiol-containing substances, as shown in Table 3. Also, INT staining, like nitroprusside staining, was inhibited if frozen sections were first incubated at pH 7·4 for 20 minutes in 0·1 M solutions of iodoacetate, *o*-iodosobenzoate or N-ethylmaleimide (Friedmann, 1952), and washed in buffer-Ringer before incubation in 0·1 per cent. INT solution.

TABLE 3. *Reactivity of INT towards various sulphur-containing compounds*

Reaction with 0·1 per cent. INT in 0·05 M phosphate at pH 7·4

Compound	*Temperature*	*Results*
Hydrogen sulphide	20° C.	Reduction to red formazan
Cysteine	"	"
Glutathione	"	"
Cysteinylglycine	40° C.	"
Benzylmercaptan	"	"
Mercaptopropanol	"	"
Thio-*o*-benzoic acid	"	"
Penicillamine ethyl ester	"	"
Reduced wool	"	Formation of red wool
Benzyl disulphide	"	No reduction
Phenyl disulphide	"	"

Treatment of frozen sections of sheepskin with 5 per cent. formol-saline (figs. 4, D and 4, F) or buffer-Ringer at 90° C. destroyed the enzymic reduction but not the sulphydryl reaction of the pre-keratinous zones of the wool follicles and Malpighian layers. Similar results were obtained with isolated wool roots.

The sulphydryl groups in paraffin sections also reacted with INT. The paraffin sections of adult sheepskin were brought down to water and treated with 0·1 per cent. INT buffered at pH 7·4. Warming of the slides on a hot plate was necessary to facilitate the reaction (fig. 3, D). The intensity of staining in the sulphydryl zones was also intensified by increasing the pH to 9·0 or 10·0. As shown with frozen sections, staining was inhibited by pre-treatment with iodoacetate, *o*-iodosobenzoate, N-ethylmaleimide.

Skin from other mammals

Frozen sections were prepared from fresh samples of adult mouse, rat, guinea pig, rabbit, and human skin, and incubated in INT with and without added succinate, as in experiments with adult sheepskin.

In all these skins the staining pattern after 5 minutes' incubation was imilar to that observed with sheepskin (fig. 5, C and D), but the enzymic re-uction occurred more rapidly, especially in mouse and rat skin.

Foetal sheepskins of all ages also reduced INT in the presence of succinate, ven before the development of epidermal plugs. Frozen sections from foetal kin in which fibres had not formed and from skin in which fibres were about o emerge reduced INT-succinate. In the latter the pre-keratinous zone in the ortex of an early keratinized fibre was readily stained as soon as it formed.

ffect of pH on enzymic-INT reduction

When frozen sections of sheepskin were incubated with succinate and INT t pH values between 3·1 and 10·0 the enzymic staining was most intense at H 8·0 and was not observed below 6·5 or above 10·0. Staining due to ulphydryl groups appeared above pH 5·5, and was intensified as the pH ncreased to 10·0.

stimation of succinic dehydrogenase activity

Succinic dehydrogenase activity in frozen sections of sheepskin and kidney ortex was estimated by a procedure resembling that of Black and Kleiner 1951). In the present experiments 0·1 per cent. INT was used as hydrogen cceptor, 0·04 M succinate at pH 7·4 as substrate, and the formazan produced fter 10 minutes' incubation at 37° C. was extracted with acetone and estimated pectrophotometrically at 465 mμ. The activity was expressed in terms of g. INT formazan/mg. dry wt. of fat-free tissue. The succinic dehydrogenase ctivity of kidney (190) as compared with skin (6·0) illustrates the lower ctivity of the latter tissue. This is partly due to the lower ratio of active to nactive tissue in skin. There was no granular enzymic deposition of formazan the control kidney or skin sections when examined microscopically although e value for the skin control (1·8) was high, owing to the sulphydryl groups the wool follicles.

Vadi' oxidase in sheepskin

The 'Nadi' oxidase reaction is used not only to indicate oxidative ability in ells generally but also for the histochemical detection of cytochrome oxidase Glick, 1949).

It was applied to sheepskin to enable the distribution of the Nadi-reactive ubstances to be compared with that of dehydrogenases. Frozen sections were nmersed for 15 minutes at 20° C. in buffered Nadi reagent and then washed buffer-Ringer at pH 7·4 and examined microscopically after mounting in per cent. potassium acetate.

The distribution of staining was very similar to that observed for dehydro-enase activity (figs. 1 and 2, C), maximum density of blue indophenol granules eing found in the wool root bulb and extending above the bulb constriction the proximal third of the follicle in both the growing wool fibre cells and the outer root sheath. This is the region of the outer root sheath which was show by Durward and Rudall (1949) to be surrounded with a network of fine capillaries. In the remainder of the Malpighian zones of the follicle and epidermi the staining was only light blue and non-granular. The sebaceous glands wer stained deep purple. The presence of 0·005 M sodium azide, which inhibit cytochrome oxidase, almost completely inhibited the Nadi staining. Application of Nadi reagent to kidney cortex produced rapid staining in the rena tubules but failed to stain the glomeruli. The distribution of the reaction wa thus identical with that of succinic dehydrogenase (fig. 5, E). The reaction wa again almost completely inhibited by 0·005 M sodium azide.

DISCUSSION

Experiments with tetrazolium salts by other workers have been carried ou predominantly with kidney and liver tissue, which reduce these reagents mor rapidly than does skin tissue. This may be due to the higher metabolic rate o kidney and liver tissue or the metabolism and respiration of these tissues ma proceed at a lower level of redox potential than in skin. Permeability of th cells to reagent and substrate, or even some specificity of the enzyme system for reaction with a particular tetrazolium, may also play a part.

In the present experiments it is shown that the little-used tetrazolium derivative, INT, is superior to other tetrazolium salts for the demonstration o dehydrogenase distribution in skin and kidney tissue. The outstandin property of INT is its greater rate of reduction in the presence of a suitab dehydrogenase system. Probably this derivative has a higher redox potentia than the other tetrazolium salts and can accept hydrogen at more than on stage in hydrogen or electron transport. Furthermore, the use of frozen section increases the rate of diffusion of the reagent and substrate into the cells, thu producing a more rapid and uniform deposition of the formazan, whic appears to be very insoluble. The increased rate of reduction of INT, on addition of DPN and ATP to skin sections, suggests that these or related co-factor are depleted by diffusion from the tissue.

Under the conditions used, the demonstration of succinic, α-glycerophosphoric, lactic, and malic (fumaric) dehydrogenases in sheepskin is established Fumarate probably reduces INT after conversion to malate in the Kreb cycle, whereas the phosphorylated intermediates may react directly with specific dehydrogenase or through the glycolytic cycle. The observation tha the rate of reduction of INT in skin is increased by succinate more than b other substrates is in accord with the work of Barron, Meyer, and Miller (1948 who found the greatest uptake of O_2 by rat skin slices in the presence of succinate. However, the INT method in conjunction with the Nadi reaction ha demonstrated the occurrence of more dehydrogenases in skin than could b concluded from the experiments of Barron and others, and provides evidenc for the existence of the Krebs cycle in epidermis and hair follicles. The increase in the rate of reduction of TTC by skin in the presence of cystein glutathione, and ascorbic acid is attributed to activation of sulphydryl enzyme n the Krebs or glycolytic cycles, which are inhibited by sulphydryl-coupling agents.

Bullough (1952) considers that epidermal cells store energy which is derived via the Krebs cycle from oxidation of carbohydrate substrates rather than from glycolysis. The energy is stored in the form of pyrophosphate bonds and accumulates until sufficient is available for mitosis. This view probably applies equally well to the undifferentiated and growing epidermal cells of the hair matrix. Thus in the wool follicle part of the Krebs cycle is active and the oxygen supply is probably adequate because of the capillary circulation to the papilla and round the follicle. Evidence for the occurrence of cytochrome oxidase in these regions has also been presented. The dehydrogenases related to the glycolytic cycle may also be necessary for the absorption and transport of metabolites in the hair bulb.

Although the cells of the epidermis undergoing mitosis exhibit considerable dehydrogenase activity, in conformity with the views of Bullough (1952), the outer root sheath also possesses similar activity although the mitotic rate is low. In this region therefore there is no simple relationship between mitosis and dehydrogenase activity. The occurrence of dehydrogenases and the previously recognized existence of phosphatases and glycogen in the outer root sheath (Johnson and Bevelander, 1946; Hardy, 1952) further suggests that this region may have a role in the transfer (and storage) of metabolites to or from the growing and keratinizing fibre. A similar distribution of dehydrogenases and phosphatases exists in kidney tubules where the latter enzyme has been correlated with resorption processes (Moog, 1946).

A further important characteristic of INT is its ease of reduction by sulphydryl compounds. It can be used in neutral solution to demonstrate sulphydryl groups in the pre-keratinous zone of wool and hair follicles and in the *stratum granulosum*. Unlike the nitroprusside and the Prussian blue reagents, INT is best applied to unfixed frozen sections under conditions which do not distort the tissues. Moreover, the red sulphydryl staining is readily distinguished from purple staining due to dehydrogenase activity.

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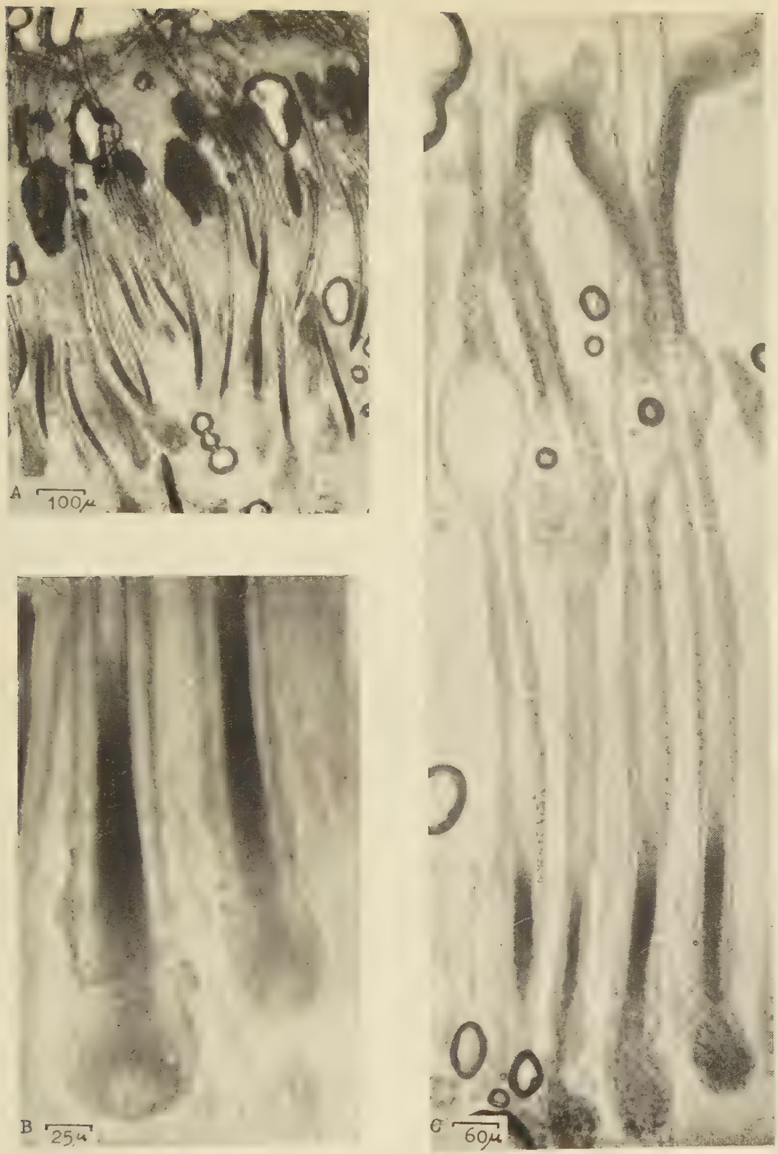

Fig. 2

G. E. ROGERS

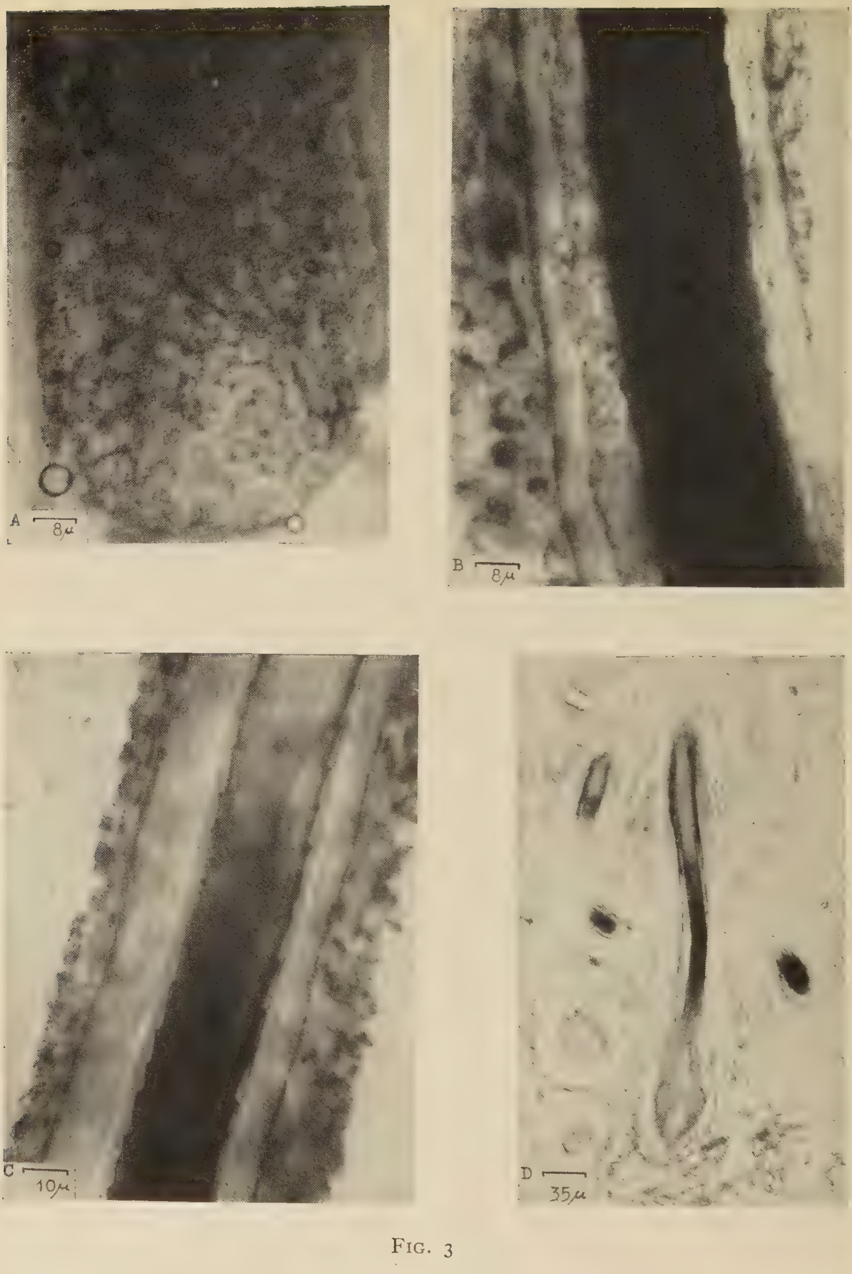

FIG. 3

G. E. ROGERS

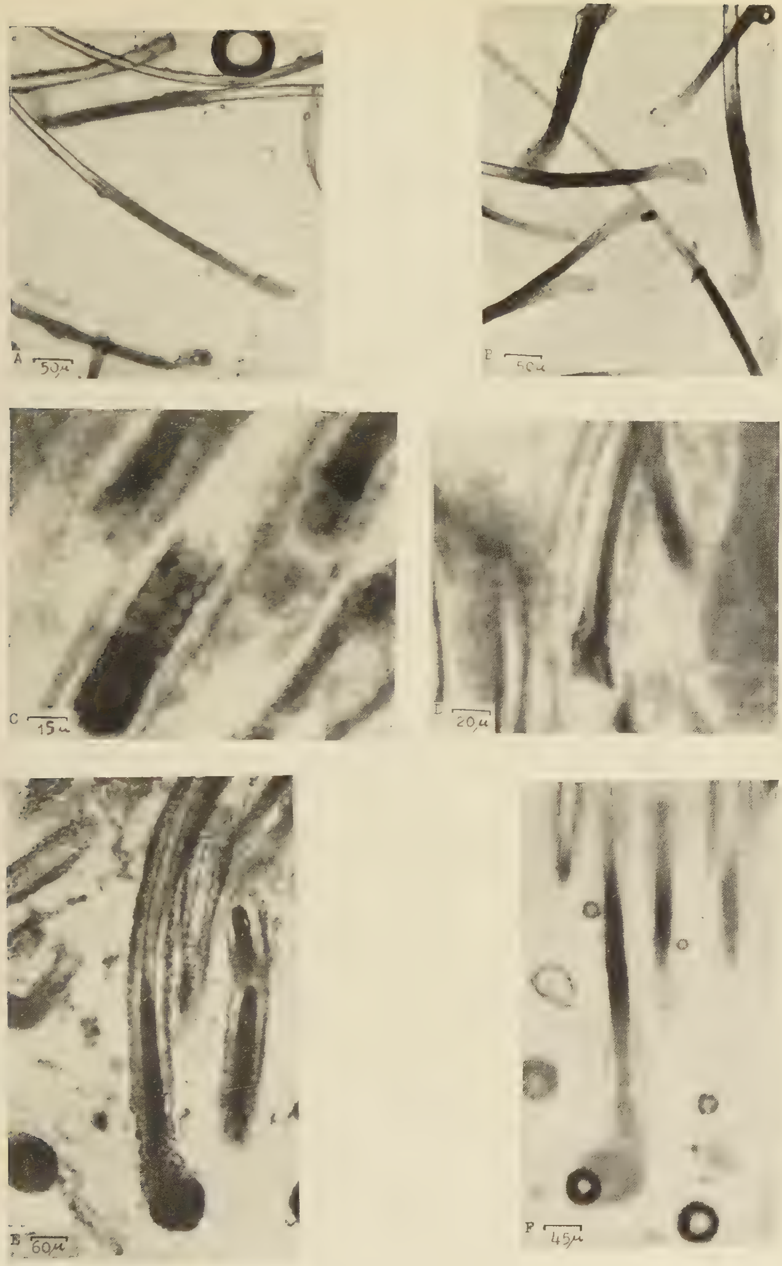

Fig. 4

G. E. ROGERS

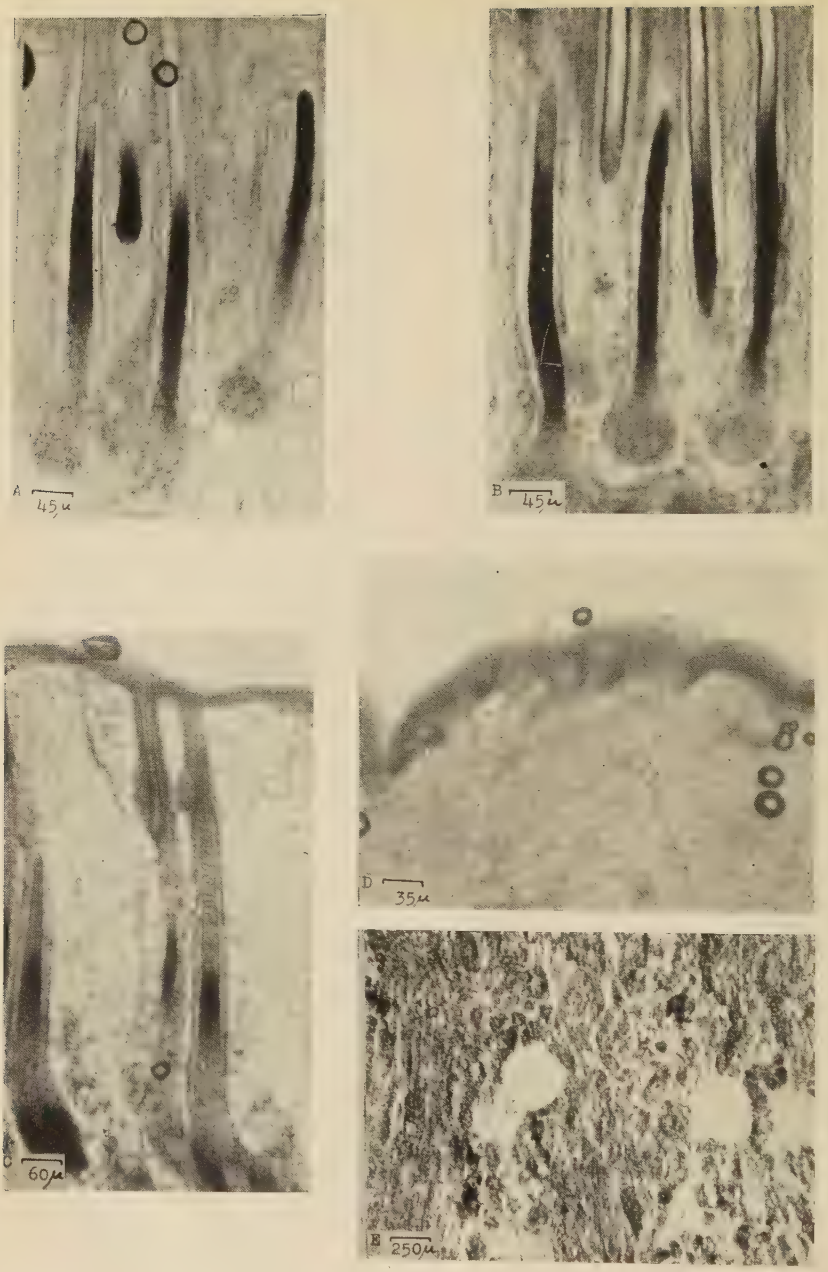

FIG. 5
G. E. ROGERS

Cytological Studies of the Acinar Cell of the Pancreas of the Mouse

Part II. The Argentophil, Osmiophil, and Sudanophil Substance

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With one plate (fig. 1)

SUMMARY

It is shown by the Aoyama, Mann-Kopsch, and sudan black techniques that there a definite series of changes in the pancreas acinar cell of the mouse after the sub-utaneous injection of a pre-determined optimum dose of neutral red. These changes re similar to those observed in the fresh tissue. In the fixed preparations they are haracterized by the appearance of argentophil, osmiophil, and sudanophil bodies aving the position, number, general configuration, and relative size of the neutral ed granules and aggregates. It is further observed that there is a shift of this substance om an apical position in the cell to a more basal one, apparently in response to the resence of neutral red.

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INTRODUCTION

IN an earlier paper (Morgan, 1953), it was shown that after the subcutaneous injection of a pre-determined 'optimum' dose of neutral red into a mouse, ere could be observed over the course of the next 12 to 15 hours, a definite ries of changes in the pancreas acinar cell in which neutral red granules eveloped, later took the form of aggregates, and still later disappeared from ew. It was also established that the neutral red granules were confined to e regions of the cytoplasm between the nucleus and the basal zymogen

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granules and for a short distance along the sides of the nucleus. Further, i was concluded that this neutral red granule cycle was a physiological proces on the part of the cell by which one of its components, possessing an affinit for neutral red, appeared to concentrate it in droplet form, and by some metho not understood effected removal of this foreign substance from the cell.

During the course of this study it was decided to apply several of th classical techniques for demonstrating the so-called Golgi apparatus to thi material, to determine what relationship, if any, existed between the neutra red granules and aggregates and the Golgi apparatus. Initial preparation (Aoyama and Mann-Kopsch) of a pancreas from an animal killed a short tim after the injection of the optimum dose of neutral red, showed a rather well-formed Golgi network in the usual location, apical to the nucleus and seemingly interwoven amongst the zymogen granules. In addition, however, ther were argentophil or osmiophil bodies having the position, number, genera configuration, and relative size of the neutral red granules and aggregates see in the fresh tissue. Further, these were the only two objects in the cell tha showed reduction of the silver or osmium.

This was a finding of considerable interest, since previous experiments i which these techniques were applied to the pancreas from mice not injecte with neutral red had never shown argentophil or osmiophil material in these secondary positions. Since they were the two most prominent objects in the cell in which reduction had occurred, and since the more basally locate argentophil or osmiophil bodies were present only after the injection o neutral red, the possibility was considered that the substance forming the so-called Golgi network and that forming the externum of the neutral re granules were the same. It was also considered that if this were true, perhaps after the injection of neutral red there was a shift of that substance from the region of the Golgi network to that of the neutral red granules—and perhaps, if a large enough dose of neutral red was given, the whole of the material o the Golgi apparatus would be used up.

Accordingly, a new series of experiments was carried out. The neutral re granule cycle was followed, tissue being fixed for the Aoyama, Mann-Kopsch, and sudan black techniques at various intervals after the injection of the optimum dose of neutral red.

Since in recent years a new outlook on the Golgi element has been presented by Hirsch, Baker, Thomas, and others, in which it is conceived as a system o non-vacuolated or vacuolated bodies, it was important that in addition to the classical Golgi methods, another technique, Baker's sudan black, should be applied to tissue in which the neutral red granule cycle could be observed.

It is the purpose of this paper to report the results of those experiments an to discuss the relationship of the neutral red granule to the Golgi apparatus

Materials and Methods

These experiments were confined to adult male mice weighing 25 to 35 gm. taken from a stock of mice supplied with food up to the time of injection

They were injected subcutaneously on the sides or back with the optimum dose of neutral red (about 0·2 mg. of neutral red per gramme of mouse body-weight) and killed by a blow on the head.

The pancreas was quickly removed from the body and bits of it measuring 2 mm. to 4 mm. in greatest dimension were cut from different parts and placed immediately in the fixative.

Two of the classical methods for demonstrating the Golgi apparatus were employed in this study. These were the Aoyama and the Mann-Kopsch techniques. These will be described briefly, since certain minor variations have been introduced.

In the Aoyama technique, bits of pancreas were placed in the Aoyama fixative (cadmium chloride 1 gm., neutral formalin 15 c.c., and distilled water 85 c.c.) for 4 hours, washed twice quickly with distilled water, and left in 1·5 per cent. silver nitrate for 15 hours. At the end of that time the specimens were again washed twice quickly in distilled water and put into the Aoyama reducer (hydroquinone 1 gm., anhydrous sodium sulfite 0·25 gm., neutral formalin 15 c.c., and distilled water 85 c.c.) for 5 hours. They were then washed ½ hour in running water and left in 50 per cent. alcohol overnight. The following day they were passed through 70 per cent. alcohol (2 hours), ethylene glycol monoethyl ether for 3 hours, and left in ester wax at 48° C. overnight. After embedding, sections were cut at 5 μ. Slides were taken through xylene and alcohols to water and left in a 0·1 per cent. aqueous solution of gold chloride for 20 minutes. They were then washed in distilled water, placed in a 5 per cent. sodium thiosulfate solution for 5 minutes, and washed in running water for a similar period. After this they were passed through alcohols to absolute alcohol and stained with acid fuchsin, according to the Altmann technique, and differentiated with picric acid alcohol solution. It may be said in passing that the application of acid fuchsin to these sections of pancreas is one which can be highly recommended, for it stains the zymogen granules and nucleoli, thus facilitating orientation of the various cell components.

Tissues for the Mann-Kopsch technique were placed in the usual fixative (saturated aqueous mercuric chloride 2 vols., osmium tetroxide (2 per cent.) 1 vol., and distilled water 1 vol.) for 24 hours, washed in two changes of distilled water, and left in 2 per cent. osmium tetroxide for 3 to 5 days at 37° C. They were changed to distilled water at this temperature and left for an additional 24 hours, then washed 3 hours, and left in 50 per cent. alcohol overnight. The following morning the specimens were passed through 70 per cent. alcohol (2 hours) and ethylene glycol monoethyl ether (3 hours) and left in ester wax overnight at 48° C. Sections were cut at 5 μ. The use of ester wax as the embedding medium is of considerable advantage in this technique, for it tends to prevent crumbling of the tissue and this makes sectioning easier. In the author's experience sectioning is often difficult with paraffin-embedded material, for tissue which has undergone prolonged osmication tends to become very brittle.

The sudan black technique employed was that modification of his original method described by Baker in 1949.

Control tissue from an animal not receiving neutral red was always included in these experiments for the dual purpose of providing normal material for comparative studies and for ensuring that the technique had been handled properly.

TERMINOLOGY

Although it is appreciated that the original description by Golgi was confined to a structure in a nerve cell, the results of countless studies of other cells since the time of Golgi represent a bulk of evidence which the present author cannot disregard, and from which he concludes, at least for the present, that they indicate to some extent homologous components in different cells. Therefore, until the time when this component or substance can be expressed in terms of its chemistry, the retention of the word Golgi seems justified.

Accordingly, the author understands the Golgi *apparatus* (or *network*) in the case of the pancreas acinar cell to be that figure shown by the classical Golgi methods as an argentophil or osmiophil network located apically to the nucleus, seemingly interwoven amongst the zymogen granules. On the other hand, the author's use of the term Golgi *substance* has a different purpose. The word 'Golgi' serves to indicate that it is the argentophil or osmiophil component of the cell; the word 'substance' is used to indicate that, although this component may perhaps at times take the form of a network, such is not necessarily the case in all circumstances. In this regard, the reason for substituting the word 'substance' for 'apparatus' or 'network' will become clear later when the changes in the argentophil or osmiophil material are described.

OBSERVATIONS

Although the findings in the pancreas of animals injected with neutral red are rather similar after the application of the three techniques used in this study, the same does not hold for tissue from a normal feeding animal that has not been injected. It is necessary, therefore, first of all to describe the pancreas of an uninjected animal as it is seen after the application of these three techniques.

The Aoyama technique

The most prominent object in the pancreas acinar cell which has been exposed to silver nitrate for 15 hours is the Golgi apparatus. This takes the form of a heavy argentophil network located apically to the nucleus, seemingly interwoven among the zymogen granules. Argentophil material is not observed elsewhere in the cell. (See fig. 1, A.)

The Mann-Kopsch technique

In tissue which has been left in osmium tetroxide at 37° C. for 3 days, osmiophil material is small in amount and usually takes the form of black ranules scattered among the zymogen granules. Some cells may show a thin r poorly formed osmiophil network. In cells that have undergone exposure osmium up to 7 days a gradual increase in the size of the osmiophil network, ften obscuring the granules seen at 3 days, may be observed. The finally eveloped Golgi apparatus as shown by this method occupies a position apical the nucleus, and, as in the case of Aoyama preparations, appears to be terwoven among the zymogen granules. (See fig. 1, c.)

he sudan black technique

Preparations of uninjected pancreas to which this method is applied show xtremely little sudanophil material in the acinar cells. Its morphology has een previously described by Baker and is confirmed by the present author as ppearing to take the form of partial or complete sheaths about certain ranules in the apical portion of the cell which cannot otherwise be differentited from adjacent zymogen granules. In the pancreas from an uninjected nimal the sudanophil material is confined to this region.

bservations in preparations of pancreas from an animal injected with the optimum dose of neutral red

Half an hour after the injection of neutral red, the Aoyama preparations how a Golgi network in its usual position, but perhaps slightly less in amount. n Mann-Kopsch material, tissue subjected to short osmication (3 days) emonstrates osmiophil granules among the zymogen granules, whereas in hat which has undergone long osmication (7 days) the Golgi network is bserved. Sudanophil sheaths are still discernible in the sudan black preparaions. In addition, all three reveal a small number of isolated, spherical, omogeneously black granules located just basally to the zymogen granules nd along the sides of the nucleus.

One hour after injection only a few strands of the Golgi network are shown y the Aoyama and Mann-Kopsch techniques and less sudanophil material is bserved among the zymogen granules in the sudan black preparations. The asal granules have increased in number and size and their configuration is ss spherical than that seen in the half-hour preparations.

At one and one-half hour after injection the typical Golgi network is no nger demonstrated by the classical methods, and sudanophil material is not dentified among the zymogen granules. In all preparations, the more basally cated argentophil, osmiophil, and sudanophil bodies are now larger.

A similar picture is observed in subsequent stages until about 8 hours after njection, the only modification being an increase in number and size of the espective bodies having the position, number, general configuration, and elative size of the neutral red granules and aggregates. (See fig. 1, B and D.) n these larger bodies, the general configuration of the neutral red granule ggregate is preserved and they appear as finely nodular, homogeneously ense, irregular objects more often elongated than round. At no time during his period is the Golgi network present.

In the advanced stages after injection of neutral red, the bodies may numbe 10 to 20 in a cell and occupy a considerable portion of its cytoplasm. In comparison to the amount of argentophil, osmiophil, and sudanophil material seen in the normal cell, the amount present at this time appears to be much greater In the sudan black preparations this difference is particularly marked.

About 12 to 15 hours after injection of neutral red, the number of argentophil, osmiophil, or sudanophil bodies is visibly less; however, the Golgi apparatus is still absent.

At 15 to 16 hours after injection, the Aoyama and Mann-Kopsch preparations again demonstrate a typical Golgi network, and sudanophil sheaths are observed about some of the zymogen granules in the sudan black slides. The argentophil, osmiophil, and sudanophil bodies are no longer present.

There is still another finding, observed in all preparations between the times when the Golgi network or sudanophil sheaths disappeared and reappeared in their usual form and positions. This took the form of a thin, irregular stringy network, often just barely discernible, which extended from the region of the basal zymogen granules to that of the argentophil, osmiophil, or sudanophil bodies. Very often this appeared to touch or connect some of these bodies in the cell. From its position and form it is clearly differentiated from the typical Golgi network. Its significance, however, is not at this point understood.

Discussion

It has been confirmed by the Aoyama and Mann-Kopsch techniques, that as in the fresh tissue, there is a definite series of changes in the pancreas acinar cell after the injection of an optimum dose of neutral red. Further, it has been shown that these changes are distinctly related in position, form, and time to the development of the neutral red granules and aggregates and their ultimate disappearance from the cell. Finally, these changes appear to involve a movement of argentophil or osmiophil substance from the Golgi region to that of the neutral red granules and aggregates at the expense of the Golgi apparatus.

Fig. 1 (plate). All four photomicrographs represent sections of the pancreas of the mouse. The word 'control' means that the animal had not been injected with neutral red.

A, Aoyama; control. The dark, argentophil network is seen in the apical part of the cells.

B, Aoyama; from a mouse killed 7 hours after the injection of neutral red. An acinus is seen near the middle of the photograph. Note the basal position of the argentophil bodies, and their configuration. The Golgi network seen in A is absent.

C, Mann-Kopsch; control. An acinus is seen near the middle of the photograph. A dark, osmiophil network is present in the apical parts of the cells, seemingly interwoven among the zymogen granules. Osmiophil bodies are not present in the basal portions of the cells.

D, Mann-Kopsch; from a mouse killed 3 hours after the injection of neutral red. In the acinus near the centre of the photograph osmiophil bodies are present at the periphery of the regions of the zymogen granules and to the sides of the nuclei. The Golgi network observed in C is absent.

It may be of interest to the reader to compare these photomicrographs with those of the living cell given in the earlier paper (Morgan, 1953).

Neutral red

Gatenby (1931) has pointed out that neutral red, when in contact with certain substances such as mercuric chloride, a frequent component of tissue fixatives, is precipitated, and he emphasizes the precaution which must be employed in drawing conclusions about the nature of 'black staining lumps in neutral red-stained corrosive osmic-fixed cells'. In fact, Gatenby considered these bodies to be artifacts. Although this objection may well be valid in some circumstances, it would not appear to affect the interpretation given to the argentophil or osmiophil bodies in the present study.

The only component of the Aoyama technique which precipitates a 1 per cent. solution of neutral red is the Aoyama reducer. It is true that both a 1 per cent. and 0·001 per cent. solution of neutral red are precipitated by the saturated mercuric chloride used in the initial fixation in the Mann-Kopsch technique. In the sudan black technique, although similar dilutions of neutral red are not precipitated by the formaldehyde-saline to which tissue is first exposed, only the 1 per cent. neutral red is precipitated by the 5 per cent. dichromate formaldehyde and the potassium dichromate subsequently employed.

The Aoyama and Mann-Kopsch techniques

(1) It was fundamental to the investigation of the theory presented earlier in this paper—namely, that the substance forming the so-called Golgi apparatus shown by the classical techniques and that forming the externum of the neutral red granule were the same or similar and that after the injection of neutral red there was a shift of that substance from the Golgi region to that of the neutral red granules, at the expense of the Golgi apparatus—that classical Golgi methods be employed, since they are the only techniques by which the Golgi substance appears as a network, the figure generally accepted as the apparatus described by Golgi.

(2) Control tissue from an uninjected animal was included in each series of experiments to reveal any 'capriciousness' of the methods used. In the Aoyama preparations, with a period of silvering of 15 hours, the control tissue always showed a Golgi net. In the Mann-Kopsch experiments a similar result was obtained.

(3) The methods, as described in the section on *Materials and Methods*, were rigidly adhered to.

(4) It was suggested that perhaps there was not actually a movement of argentophil or osmiophil substance from the Golgi region to that of the neutral red granules, but that the neutral red granules had a greater affinity for the silver, and that there was not enough silver left to form the Golgi apparatus, or that the 15-hour period of silvering was insufficient for its demonstration. Accordingly, two lots of tissue from an animal injected with the optimum dose of neutral red 5 hours previously (a period at which previous preparations had shown the argentophil bodies but not the Golgi apparatus) were carried through the Aoyama technique with the following variations:

after the initial 4-hour period of fixation, one lot was left in 1·5 per cent. nitrate for 48 hours, the old silver nitrate being replaced by new on the se day. The second lot was silvered for 3 days, and the silver nitrate cha daily. Subsequent examination of the finished preparations of these two s mens revealed little, if any, discernible difference in the amount of reduced. Argentophil bodies corresponding to the neutral red granule a gates were present, but the Golgi apparatus was absent.

The Aoyama preparations always showed a narrow and irregular ba tissue at the periphery of the specimen in which little or no reduction of had taken place, both Golgi apparatus and argentophil bodies being ab However, in the inner, and by far greater, portion of the tissue the fin were uniform and interpretation was neither difficult nor uncertain.

(5) It has been shown during the course of many experiments that the re obtained are reproducible. In those specimens in which advanced or maxin neutral red granule formation had been obtained, the Golgi network was n observed in any portion of the section. Conversely, at similar stages, argentophil or osmiophil bodies were seen in almost every acinar cell, the exception of the peripheral band of tissue in the Aoyama preparatio

The similarity of the neutral red granule substance and the argentophil, osmio or sudanophil substance (Golgi substance)

It is concluded from the results of the studies just presented that in acinar cell of the pancreas, that portion of the neutral red granule respons for its immiscibility with the cytoplasm, on the one hand, and the argento osmiophil, or sudanophil substance shown by the appropriate techniques the other, are similar. This conclusion is based on the following observati

(1) The argentophil, osmiophil, or sudanophil bodies are not observe the acinar cell unless the animal has been injected with neutral re

(2) Their position, number, general configuration, and relative size is of the neutral red granules and aggregates.

(3) Their time-relationships in appearance, development, and disapp ance are identical with those of the neutral red granules and ag gates.

The Golgi substance

The observation that the argentophil, osmiophil, and sudanophil bo identified in fixed preparations of pancreas from an animal injected neutral red are formed at the expense of the Golgi apparatus as demonstr by the Aoyama and Mann-Kopsch techniques and of the lipochondri shown by the sudan black method is interpreted as evidence that the subst forming the Golgi apparatus or lipochondria in uninjected tissue is simil that forming the granules and bodies seen after application of the techniques to tissue from injected animals.

The Golgi apparatus

The finding of similar results after application to tissue containing neutral ed granules of all three techniques employed in this study is interpreted as evidence, not previously available, that the sudan black method of Baker erves to identify the same substance which is responsible for the reduction of silver and osmium in tissue treated by the classical Golgi methods. Therefore, since the sudan black method has been shown to produce true pictures of objects visible in the living cell, it is concluded that, to the extent that it akes the form of a network, the Golgi apparatus is an artifact. However, by he same evidence and reasoning, proof is provided that there is a substance nitially and normally present in the cell in the position on which the Golgi apparatus shown by the classical Golgi methods is 'developed'. As often suggested in the past, the artificial Golgi apparatus is probably due to excessive deposition of silver or osmium on the primary Golgi substance.

The movement of the Golgi substance in the cell in response to neutral red

The observation that there is a gradual diminution in size of the Golgi network as demonstrated by the classical techniques as the size and number of the argentophil or osmiophil bodies increase, strongly suggests that there s a movement of the Golgi substance from the Golgi region to that of the neutral red granules and aggregates. The further observation that this diminution goes on to complete absence of any argentophil or osmiophil material in the Golgi region in the advanced formation is added evidence for this contention. Finally, the fact that at the time when the bodies corresponding to the neutral red granules and aggregates are no longer present, the typical Golgi network is again demonstrable, makes the movement of this substance in the cell almost a certainty.

The increase of the Golgi substance in the cell in response to neutral red

Previous reference was made to the observation, especially in the case of the sudan black preparations, that the amount of Golgi substance as evidenced by the argentophil, osmiophil, or sudanophil bodies in the later stages of development of the neutral red granule aggregates appeared to be considerably greater than that observed in control preparations of normal tissue. If this is true, then additional evidence is provided supporting the view, proposed in the first paper of this series, that the neutral red granule cycle represents a physiological process of the cell's reaction to the presence of neutral red. In view of the observation that the Golgi apparatus can no longer be demonstrated by the classical methods during the stages of the neutral red granule cycle when the aggregates are present, it may be further concluded that during this period all, or essentially all, of the Golgi substance in the cell s bound up in the form of neutral red granules since little, if any, is demonstrable elsewhere in the cell.

The nature of the neutral red granule

In the previous paper the neutral red granule was described as a separate spherical, reddish body which was sharply demarcated from the surrounding homogeneous pale pink cytoplasm, and it was suggested that its obvious immiscibility with the watery cytoplasm might be indicative that at least one component of the neutral red granule was of a lipoid nature. Although considerable caution must still be exercised in deciding whether the neutral red granule consists entirely of lipoid or whether it consists of a lipoid sheath surrounding a watery internum, it is permissible from the findings of experiments described in the present paper to draw some additional conclusions about the neutral red granule.

It has been shown that the neutral red granule is argentophil, osmiophil and sudanophil. From this evidence certain inferences may be drawn:

(1) The techniques used are only successful by virtue of the fact that they include special measures by which lipoid material is fixed. Conversely the Golgi apparatus as revealed by the Aoyama and Mann-Kopsch techniques and the lipochondria shown by the sudan black method are not demonstrable if the tissue has first been exposed to lipoid solvents.

(2) Of a more direct nature is the evidence that the neutral red granule is sudanophil, for sudan black only colours lipoids.

(3) The neutral red granule contains a substance which has the ability to reduce silver and osmium.

The finding that the bodies in the fixed preparations corresponding to the neutral red granules and aggregates observed in the fresh tissue are homogeneously dense without vacuolation allows consideration of the possibility that the neutral red granule possesses a lipoid externum, but similarly it could indicate that the entire substance of the granule apart from the neutral red consisted of lipoid. Therefore, evidence is not available at present showing that there is a second component, an internum, of a watery nature. The presence of lipoid in the neutral red granule is, however, established.

The work of Chlopin (1927), in which neutral red was also used, with the discovery of his 'Krinom' substance after the application of basic dyes to tissue containing neutral red, would appear to be directly related to our own. The consideration of Chlopin's 'Krinom' and the effects of a variety of fixatives on the neutral red granules and aggregates will form the basis of another paper.

I wish to express my appreciation to Dr. John R. Baker for his help and continued interest in this study. Professor A. C. Hardy, F.R.S., kindly provided facilities which made the work possible.

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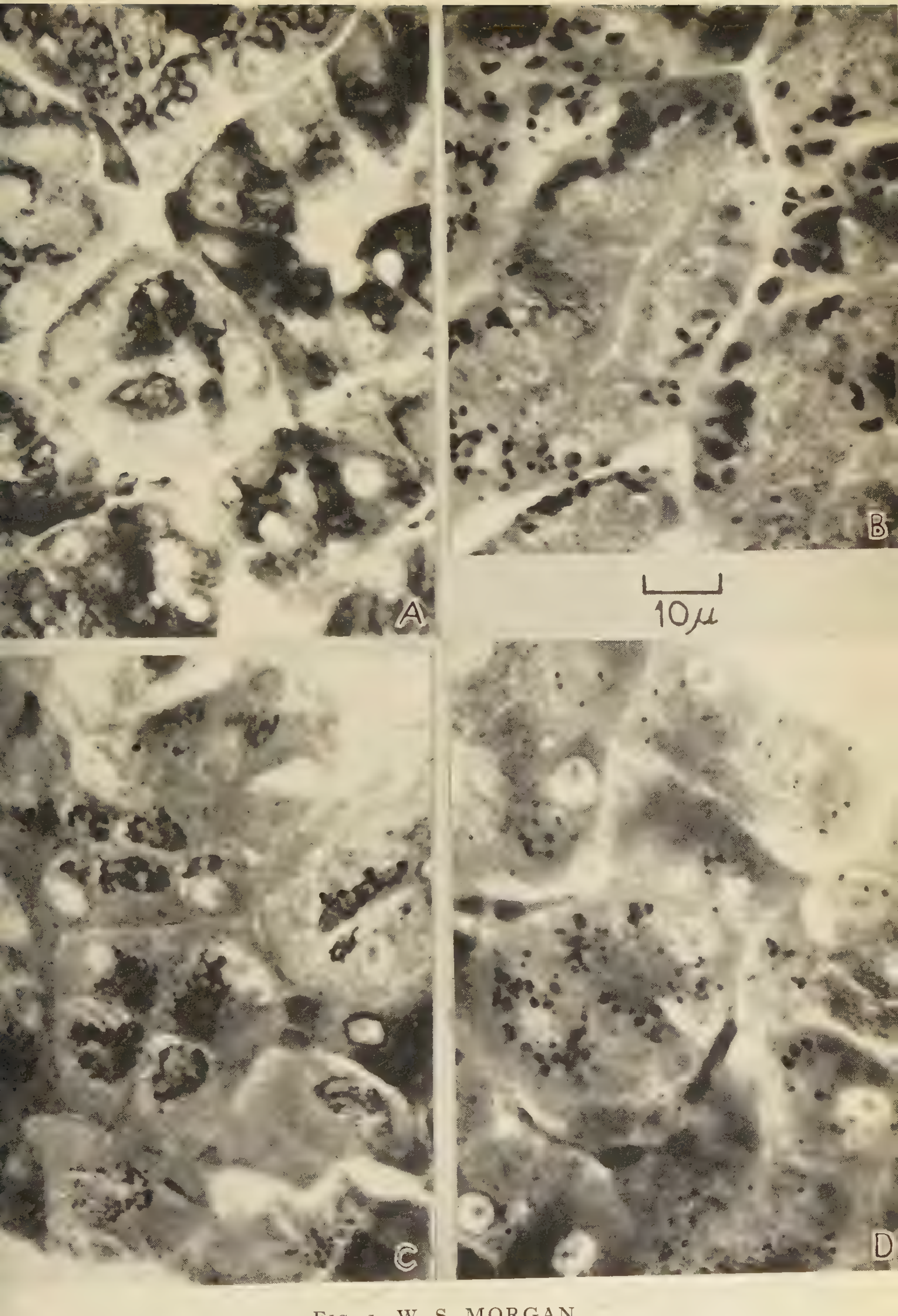

FIG. 1—W. S. MORGAN

The Relationship of the 'A' and 'B' Cells of the Pancreatic Islet Tissue of the Grass-snake (*Natrix natrix*)

By E. J. W. BARRINGTON

(*From the Department of Zoology, The University of Nottingham*)

With one plate (fig. 1)

SUMMARY

An account is presented of some cytological characteristics of the pancreatic islet tissue of the grass-snake (*Natrix natrix helvetica*), based upon supra-vital observations and upon the application of different techniques of fixation and staining. It is concluded that the so-called 'A' and 'B' cells contain granules of essentially the same type, and that they are stages in a single secretory cycle. It is suggested that the 'A' cells constitute a relatively inactive storage phase, while the 'B' cells are concerned with the formation and discharge of the final secretory product, free phospholipine becoming demonstrable in association with the granules during this latter phase.

INTRODUCTION AND PREVIOUS WORK

IN a previous publication it has been argued (Barrington, 1951) that the activity of the pancreatic islet tissue of the frog centres round a characteristic 'specific granule', and that the apparently distinctive cell types are stages in a single secretory cycle, the differences in their staining reactions depending largely on differences in the stages of development of this granule. It was there pointed out that the frog's islets were admittedly anomalous in certain respects, and that before it became possible to generalize from this argument it would be necessary to extend the work to islets of a more conventional pattern in which cells of the classical 'A' and 'B' types had already been claimed to exist. Such an extension is the purpose of the present work, the animal selected being the grass-snake (*Natrix natrix helvetica*). This choice has been made because an interesting contradiction already exists in the literature dealing with reptilian islets. Laguesse (1902), in his pioneer studies, devoted considerable attention to various snakes, the grass-snake among them, and concluded that the granules of their islet tissue cells were all of one type, differing merely in their stage of development. Subsequently, the concept of contrasted cell types became firmly established in the literature, and Thomas (1942) has described the islets of a number of species of snakes as being composed largely of 'A' and 'B' cells, with a small proportion of 'D' cells in addition, distinguishable by their reactions to the Azan staining method, and fully comparable with the similarly named cells which he had previously described in a number of mammals and selachians (1937, 1940). It is hoped to achieve here some reconciliation of these two points of view and, with particular

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reference to the 'A' and 'B' cells, to examine how far the conditions in th islets of the snake admit of the same interpretation as that already suggeste for the frog.

MATERIAL AND METHODS

The animals used were obtained from a dealer and doubtless varied in thei condition and nutritive state. Laguesse remarked on the extent to which th appearance of the islets of snakes was influenced by the degree of fasting which the animals had experienced, but such variations do not affect th evidence to be presented here. The main body of the islet tissue is conveniently located in large masses at the splenic end of the pancreas, where in fact, they are readily observable with the naked eye, and small portions o this region were fixed in Bouin's fluid, Helly's fluid (with and without postchroming), and Helly-osmic (ten parts of Helly's fluid and one part of 2 pe cent. osmium tetroxide). Sections, cut at 3 μ to 5 μ, were stained by the Azan method, Heidenhain's iron haematoxylin, Gomori's (1941) chromium haematoxylin and phloxin (devised to differentiate the 'A' and 'B' cells of mammalian islets), and Kull's mitochondrial method (acid fuchsin toluidine blue, and aurantia). Both frozen and wax sections were coloure with sudan black (Baker, 1949; Thomas, 1948), while material was also carrie through the acid haematein and pyridine extraction techniques as a test fo phospholipines (Baker, 1946, 1947*b*). For supra-vital observations the tease tissue was examined in 0·7 per cent. sodium chloride with added calcium chloride (Baker, 1944) and was stained with Janus green B at a concentratio of 1 in 10,000.

OBSERVATIONS

Supra-vital material

Studies of pancreatic islet tissue have tended more and more to be base upon the polychromatic staining of fixed material. Since, however, as will b shown below, different techniques may yield substantially different pictures it is very difficult to interpret the results without having some independen standard of reference, and it is therefore proposed here to begin with description of the living material. When this is teased and examined in norma saline the islets are readily distinguishable as a result of their finely granula appearance, and it is possible to agree with Laguesse (1902) that they ar composed, broadly, of two types of cells, those 'à grains simplement fins' an those 'à grains très fins'. These two will be referred to here for convenience a 'A' cells and 'B' cells, although it must be made clear at the outset that whil the majority of the islet cells can readily be placed in one or other category there are intergradations between them, together with what are probabl developmental stages. The granules of the 'A' cells are variable in size, but ar predominantly large, being often well within the range of size of at least th smaller zymogen granules. These larger ones are more or less spherical, an have a characteristic appearance resulting from their differentiation into a opaque cortex, greyish by transmitted light, and a more translucent medull maller granules are also present in these cells, particularly towards their istal ends; they grade into those of the 'B' cells, from which they cannot, in ıct, be differentiated, and there is no doubt that the appearance of such pre- arations supports the view of Laguesse that the two types of cell represent ıerely two stages in development, although it will be necessary to disagree ı part with his interpretation of the actual relationship of these stages.

These smaller granules are often irregular in shape, and seem at first sight ; though composed only of the cortical material. Close examination and care- ıl focusing of them shows, however, that they often possess a curvature hich suggests that this material, like that of the larger granules, is associated ith a spherical medullary component; it may be supposed that the latter are ifficult to resolve because of the irregular shape of the cortical substance, though it is quite possible to focus clear circular areas which could mark ıeir positions. Such indications are no more than suggestive, but it will be ːen below that there is much to support this interpretation.

Bensley (1911) showed that the granules of the islet tissue of the guinea-pig ıd toad could be stained supra-vitally by Janus green B, and this is true also ' the present ones. The staining of the snake pancreas is very uneven and ratic, doubtless because the dense structure of this organ hinders the diffu- on of the dye, but in successful preparations the mitochondria of the zymo- ːn cells are intensely stained, appearing often as thick filaments which may ıow a characteristic club shape. Both types of islet granules are also stained g. 1, A) although less intensely, and it can be seen that the dye has a parti- ılar affinity for the cortical material, the medullary region of the larger 'anules being stained only faintly or not at all, so that they sometimes have .e appearance of rings, as may be observed in the 'A' cell shown in the ːntre of fig. 1, A. This suggests, of course, some difference in composition of .e two components, and reinforces the view that the 'B' granules have some- ing in common with the cortical component of the larger 'A' ones. No struc- res other than these granules have been observed to be stained by Janus een B in the islet cells.

xed material (*frozen sections*)

While supra-vital observations certainly aid in the interpretation of the ːed and stained cells, it has in practice proved very difficult indeed to bridge th certainty the gap between the two categories of observations, the prob- n being, of course, to decide as to the degree of artifact which may be intro- ıced by any particular technique. Prolonged comparison of the results tained, however, suggests that the nearest approach to the appearance of the ing cells is provided by the methods of fixation and embedding used in the id haematein and sudan black techniques of Baker (1946, 1949), which avoid th the precipitation of proteins in the initial fixation and also the action lipoid solvents in the later stages.

In all fixed material, as also in the living, a difference in the shape of the ' and 'B' cells, while by no means invariable, is very commonly seen (fig. 1, B and F), the former being elongated, sometimes with a swollen basal region i which is located the nucleus, while the latter are more truncated and li wedged between the distal ends of the others. The nucleus of these 'B' cells i often far from circular in outline, appearing crescent-shaped, for example, o presenting some irregular folding of its membrane, a condition which is suggestive of particularly active nucleo-cytoplasmic interaction.

The sudanophil reaction of the tissue will be discussed in a later publication. The granules themselves are not usually coloured, but their appearanc confirms the conclusions drawn from the living material in that the larger 'A granules are frequently seen on careful focusing to be composed of a dens yellowish cortex arranged around a much more translucent medulla, whil the 'B' granules resemble the cortical material in appearance, and can b observed to be drawn out into curved shapes which tend to run into eac other.

The acid haematein technique (fig. 1, B) reveals blue-stained granules i both types of cell. Those in the 'A' cells clearly correspond with the 'A granules already described, ranging from large ones of circular outline t smaller and more irregular ones; often they are stained throughout, bu sometimes a deeply stained cortex can be differentiated from a much lighte medulla, while in extreme cases a granule may appear as a coloured ring wit a colourless centre. It seems wholly probable that the granules in the 'B' cell in these preparations also correspond with the 'B' granules already describe Like the latter, they resemble the 'A' granules, and more especially the cortic region of the latter; they are extended into curved shapes, with sometimes th quite definite form of a crescent or ring. They seem often to be associated wit clear round areas which, as suggested above, could well represent spheric medullary components.

If this general view of the relation of the 'A' and 'B' granules be correc however, the application of the pyridine extraction control (fig. 1, C) must b held to produce a somewhat unexpected result, for while the staining of th former is not obviously affected by this procedure, most of the latter are n longer visible, as can be seen by comparing fig. 1, B and C. The histochemic

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FIG. 1 (plate). Pancreatic islet tissue of the grass-snake; for further explanation see text

A, stained supra-vitally with Janus green B; an 'A' cell is seen centrally, with 'B' cells abo and to the left.

B, acid haematein.

C, pyridine extraction, control to B.

D, Bouin's fluid and Heidenhain's iron haematoxylin.

E, Bouin's fluid and Gomori's chromium haematoxylin and phloxin; the 'A' granules a stained pink, but their cortex is differentially stained by the haematoxylin.

F, Helly's fluid, post-chromed, and Gomori's technique; the 'B' cells are much lighter th the 'A', but show distinct phloxin-stained granules.

G, Helly-osmic and Gomori's technique; the 'B' cells are much darker than the 'A'.

H, Helly's fluid, post-chromed, and Kull's technique; the 'B' cells are much lighter than t 'A', but show distinct fuchsinophil granules.

I, Helly-osmic and Kull's technique; the darker 'A' cells (upper left) show fuchsinop granules of variable size, while the lighter 'B' cells (lower left) show small ones.

implication of this is that the 'B' granules contain phospholipine, and it is necessary to consider how far this can be reconciled with the fact that the other observations so far described suggest that these granules are derived from the cortex of the 'A' granules. It is the writer's view that the simplest explanation which is in harmony with all the data to be reported here is that the granules of the two types of cell are fundamentally similar in their composition, but differ in their functional condition, and that they contain a phospholipine component which is closely bound in the 'A' granules, but free in the 'B' granules, so that only from the latter is it removed by pyridine extraction. This, however, is admittedly an interpretation which relies upon a substantial element of personal opinion, and it must be judged in the light of other observations now to be described.

Fixed material (wax sections)

In selecting staining methods for the study of islet tissue there has been a natural tendency amongst investigators to prefer those polychromatic methods which appear to give the sharpest differentiation of the supposed cell types, and Heidenhain's iron haematoxylin has been somewhat neglected. In the present work, however, it has yielded results which can be related without serious difficulty to the appearance of supra-vital material, and it will be convenient to deal with these before passing to the more commonly used methods. Staining with haematoxylin, then, after fixation in Bouin's fluid (recommended as one of the best fixatives for islet tissue: Gomori, 1939), reveals (fig. 1, D) intensely stained granules in both 'A' and 'B' cells, the latter containing in addition some ill-defined background material. The largest granules in the 'A' cells have a deeply staining cortical rim and an unstained medulla (examples are in focus at the point indicated in fig. 1, D), while the smaller ones have exactly the appearance which would be given by incomplete rims. In the 'B' cells the granules are mostly smaller than these, but they vary in size and there is no sharp distinction between the two; careful focusing discloses that they frequently display a curvature which suggests that they, too, could well be associated with unstained medullary components. Fixation in Helly's fluid, in the same fluid with post-chroming, and in Helly-osmic gives essentially the same results; after the last two, however, the 'B' granules are perhaps a little more solid and well-defined, and the background material more obvious, although still diffuse in outline.

It will be apparent that these results agree closely with the conclusions already drawn in suggesting that the 'A' and 'B' granules have a common structure and that the two categories of cells are of fundamentally the same type. It is the more remarkable, therefore, that an entirely different impression can be given when Gomori's well-known method (1941) is applied after Bouin fixation, for this appears at first sight to give a sharp differentiation of 'A' and 'B' cells (fig. 1, E), the former being loaded with large and closely crowded granules, bright pink in colour, while the 'B' cells present only an ill-defined blue granulation which on closer inspection appears to be formed by irregular threads and flocculations, variable in size and shape. Further examination, however, makes it possible to see that the results of this method amplify rather than contradict the conclusions already drawn. It is evident that phloxin stains the medulla of the ‘A’ granules, and possibly also the cortex, but the chromium haematoxylin certainly stains the latter, as may be seen by cutting down the red transmission with an appropriate filter, when the granules may appear as haematoxylin-stained rings, examples of which are seen at the point indicated in fig. 1, E. In comparing these with the similar rings seen in fig. 1, A (Janus green B) and fig. 1, D (iron haematoxylin), it should be borne in mind that in the latter two the medullary substance in the centre of the ring is unstained, whereas in Gomori preparations it is stained pink.

The condition in the ‘B’ cells is less clear, but it is quite possible to interpret them as containing background material, stained light blue, together with what are virtually ‘ghosts’ of the true granules; these consist of remains of the rim material, also staining pale blue, and often forming more or less incomplete rings which are faintly distinguishable in some of the ‘B’ cells seen in fig. 1, E, together with occasional signs of phloxin-stained medullas, which can sometimes be brought into focus within the cavities of these rings, but which are seen only sporadically and with no great clarity.

It is clear that what is needed to give support to this interpretation is a technique capable of improving the definition of the contents of the ‘B’ cells and revealing with certainty the medullary components which are here assumed to exist in them, and this is not, in fact, difficult to contrive. Fixation in Helly’s fluid, which has been extensively used in islet studies (Thomas, 1937), gives a picture essentially similar to that already described, but if the fixation is followed, before dehydration and embedding, by ‘post-chroming’ in 5 per cent. potassium dichromate for 3 days at 37° C., the desired improvement is obtained, the ‘B’ cells being now found to contain a number of clearly-defined pink granules (fig. 1, F), similar in appearance to those of the ‘A’ cells, although generally smaller in size. These ‘B’ cells contain also a blue-staining substance, irregular in form but better defined and more compact than in Bouin material, and tending to form granule-like masses of much the same size as the pink granules. Comparison with the previously described preparations makes it reasonably clear that the pink-stained ‘B’ granules are best interpreted as including those medullary components of which the existence has been inferred above although they could not with certainty be directly observed. In other words, the ‘B’ granules of Heidenhain preparations, for example, represent the more or less incomplete rims of the true granules, while the phloxin of the Gomori method reveals their medullary components. No doubt the blue-stained material seen in the ‘B’ cells in the latter type of preparation corresponds to the indefinite yellow-grey background substance of the Heidenhain preparations.

The use of the Gomori method after Helly-osmic fixation provides some additional evidence, while affording an interesting illustration of the ifficulty attending the interpretation of these polychromatic techniques. ctually, the results in this instance are somewhat variable, evidently epending on the degree of penetration of the components of the fixaive, uneven fixation being a well-known drawback of osmium tetroxide, ut it is usually possible to find areas in the section which give a quite tartling and brilliant differentiation of the 'A' and 'B' cells (fig. 1, G), and hich, in the absence of comparison with the results of other techniques, ould undoubtedly suggest complete independence of each other. The 'A' ells still exhibit pink granules, brightly coloured and sharply defined, but the B' cells are filled with irregular, granule-like clumps of dense blue-staining aterial, and the pink granules, so clearly seen in Helly-post-chromed ections, are no longer visible in most of them. In comparing these results it hould be noted that the dark 'B' cells of fig. 1, G (Helly-osmic) correspond ith the light ones of fig. 1, F (Helly-post-chromed), the darkness of the rmer resulting from the dense mass of haematoxylin-stained material which resumably represents the paler and much less well-defined material already oted in the latter, and referred to in other preparations as background aterial. Evidently, then, the true 'B' granules could well be present in elly-osmic sections but yet be obscured by the material which is collected round them, and that this is actually so is confirmed by the fact that careful earch will sometimes reveal traces of a pink stain in the centre of a clump, hile, more significantly, there can be found occasional cells showing numerus pink-stained granules around which threads of the dark material can e seen, the appearance being exactly indicative of a stage intermediate beveen the two main types of cell. Even more convincing, however, in this nnexion are the appearances obtained with the Kull technique, and it will ow be convenient to refer briefly to some results obtained with this and her staining methods in so far as they usefully supplement the above bservations.

The Azan method has been extensively used in islet studies; Gomori (1943), owever, comments on its failure to give a sharp picture of the 'B' granules, nd this is rather my experience in the present work. The 'A' granules after l of the above-mentioned fixatives are stained red or orange-red; a cortical ifferentiation is not usually seen, but it can be evoked by carrying differenation to an extreme, when granules may be found which show a stained rtex and a colourless medulla. In the 'B' cells it is possible to distinguish anules which are not sharply differentiated in reaction from those of the ' cells, but which show in general a diminished affinity for azocarmine, nding, therefore, to stain orange, orange-red or orange-grey in colour; these early correspond with those stained by phloxin, and it is of interest that they n even be seen, although ill-defined, after Bouin or Helly fixation, despite e fact that they are almost completely invisible in the same material when is is stained by the Gomori method. It is thus clear that the apparent abnce of granules in certain preparations does not necessarily mean that they e not preserved, but merely that there is a deficiency in the mordanting or in some other interaction of the particular fixative and stain employed. Thes ‘B’ granules are associated to a variable extent with an orange or blue or blue grey material which may form a reticulum around them and which tends t obscure them; presumably this corresponds with the haematoxylin-staine material of Gomori preparations, but the picture is less well defined, and, i my experience, compares unfavourably with that given by other methods.

The Kull technique has been little used in islet studies, understandabl enough, since it is not well adapted for use after such fixatives as Bouin’ and Helly’s fluids, the picture which it then presents being drab and unhelpful. Brilliant effects are obtainable, however, with Helly-post-chrome material, a sense of the fundamental uniformity of the ‘A’ and ‘B’ cells bein even more strikingly conveyed than after the Gomori technique, both type (fig. 1, H) exhibiting abundant red granules, those of the former being larg and crowded, as before, while in the latter they are smaller and more scattered. Under low-power observation there is none the less an impression o a difference in colour between the two types, and under oil-immersion this i seen to result from the fact that the toluidine blue stains a thin layer o material which appears to form a surface or investing layer around the ‘B granules, and which presumably corresponds with the haematoxylin-staine material of Gomori preparations. Comparison of the results of these tw methods makes it quite clear that acid fuchsin reveals more ‘B’ granules tha does phloxin; presumably their affinity for the latter stain is less, and it may b also that they are further obscured by the chromium haematoxylin, whic gives an impression of staining more densely than does toluidine blue. Helly osmic material (fig. 1, I) gives a very similar picture after Kull staining, wit perhaps even greater clarity, and this makes it quite evident that the absenc of phloxin-stained granules from the ‘B’ cells in Gomori preparations of th same material is again a property of this particular technique, for such cell are crowded in Kull preparations with brilliantly fuchsinophil granules, whil the toluidine blue is again much less in evidence than is the haematoxylin. I can thus be said that, broadly speaking, Helly-osmic fixation followed by th Kull staining technique tends to emphasize a common pattern in the ‘A’ an ‘B’ cells, while the effect of the Gomori method is to emphasize difference between them.

DISCUSSION

It seems clear from the present work that any interpretation of the organization of islet tissue cannot safely be based on the results obtained from the us of any one polychromatic staining technique, but must also take into accoun the results obtained from other procedures as well as the appearance of th living cells. Such an interpretation has been developed in the above accoun as a convenient basis for the exposition of the data, and it will now be well t summarize it in a concise form.

The ‘A’ cells of the snake are loaded with large granules which are differentiated into a cortex and medulla. The former is the more opaque regio n the unstained living cell, and can be selectively stained by Janus green B nd by Heidenhain's haematoxylin, while the medulla (and probably the cortex, too, although it is difficult to be sure of this) is stained by phloxin, azocarmine, acid fuchsin, and acid haematein (with or without pyridine extraction). The cortical rims may often be incomplete (an effect which is likely to be enhanced as an artifact of fixation), and this seems to be the cause of the appearance of smaller and more irregular granules which grade into those of the 'B' cells. These latter are considered to be of fundamentally the same constitution as the 'A' granules, although they are in general of smaller size and less closely packed. The acid haematein test reveals the presence of phospholipine in the 'B' cells, apparently associated with the granules, but here again the absence of any sharp distinction either of form or colour between the 'A' and 'B' granules leads to the conclusion that this phospholipine is actually present in both, but is firmly bound in the former and only removable by pyridine in the latter. It is impossible as yet to prove the correctness of this conclusion, but if it be accepted as a working hypothesis it leads in its turn to the suggestion that the two categories of granules are in two different phases of activity. Now it is generally agreed that 'B' cells are immediately concerned with the secretion of insulin, and signs of their degranulation can certainly be found in snakes which have been injected with glucose. Having regard to this, and to the general appearance of the cells, it seems reasonable to suppose that the 'A' granules, packed closely as they are within the largest cells of the islets, constitute an inactive storage phase, and that the 'B' granules are concerned in the final stages of the formation of the secretory product, the unbinding of the phospholipine being correlated with this. From this point of view the basiphil material which is found in the 'B' cells, and which is particularly evident after Helly-osmic fixation, may well be regarded as the product of the activity of the granules; it could therefore be insulin, or the immediate precursor of it, and would be protein in constitution, and this would explain why it is not observed in supra-vital preparations or after formal-calcium fixation, but becomes apparent after fixatives, such as Bouin's or Helly's fluids, which contain protein precipitants. It may be added that the association of free phospholipine with the 'B' granules would account for the beneficial effect of post-chroming and of fixation in Helly-osmic upon the staining of these, for the action of potassium dichromate is to stabilize phospholipine, while osmium tetroxide preserves lipoids in general.

On the assumption that the 'A' granules are a storage phase one would surmise that they would be composed in part of protein, to which the assumed phospholipine component might be bound, and it may therefore be mentioned here that the Sagakuchi test for arginine (Baker, 1947*a*) does actually give a weakly positive reaction in these granules. So far no reaction has been obtained from the 'B' cells, a result which is not, perhaps, altogether surprising in view of the fact that insulin is reported to contain only 3 per cent. of arginine. Conceivably, then, the test might well yield a positive result only in the 'A' cells, where, on the present hypothesis, there might be a high concentration of protein held in readiness, so to speak, for further processing, but this poin requires further examination and is not pressed at this stage.

The general interpretation advanced here implies, of course, that there should be stages transitional between the 'A' and 'B' cells, but on this point it must again be emphasized that the constant reference in this account to two contrasted cell types is a simplification adopted for convenience of description. The two are not, in fact, entirely distinct; the granules of the 'B' cells, for example, vary much in size, and grade into the 'A' granules, while, as Laguesse noted, individual cells can be seen which contain granules of both types, as judged by their size. However, the majority of the cells do admittedly fall into one or other category, and although, as mentioned above (p. 287), careful search does show occasional cells of an intermediate type, the fact that these are rare certainly suggests that at any one time a substantial proportion of the islet tissue must be inactive. It is hoped to pursue this aspect in a later publication which will attempt an analysis of the secretory cycle in this tissue; this will refer also to other cells which seem likely to prove to be developmental stages of those described here, and will take account of the 'D' cells described by Thomas (1942).

It is of interest to find that Laguesse (1902) noted that during prolonged fasting the larger granules ('A' of the present account) came to predominate more and more, and that the smaller ('B') ones were particularly abundant during digestion. His view was that the latter were developing granules and that the former were the 'ripe' secretory ones, but the observation would seem to be as well, if not better, reconcilable with the present interpretation, according to which the larger inactive granules would come to predominate during periods of fasting when the demand for insulin would presumably be reduced. Laguesse did not attempt a detailed analysis of the properties of the granules, but, as already mentioned, he insisted that they were in different stages of development and did not constitute distinct types. He concluded that the 'A' granules were more stable than the 'B', remarking in particular that Bouin's fluid either fixed the latter badly, or at least failed to mordant them. He further noted that isolated granules dissolved eventually in 1 per cent. acetic acid, but more slowly than the zymogen granules, and found that if the tissue was 'fixed' for 24 hours in 2 per cent. acetic acid, and subsequently embedded and sectioned, the zymogen material had disappeared altogether, while the islet cells contained a 'colloidal mass' staining with eosin or lightly with iron haematoxylin. It would seem possible to account for this on the interpretation suggested here by supposing that the acetic acid would weaken the structure of the granules and allow the assumed phospholipine to be dissolved out during dehydration, leaving the remainder of the granule to form the residue, which would presumably consist largely of protein.

Finally, it can be said that the present account seems to be in good general agreement with conditions in the islet tissue of the frog (Barrington, 1951), this agreement being the more striking in that it was far from obvious in the early stages of the work and has been realized in part by lines of argument hich could not then have been foreseen. It was shown that in the frog there as only one type of specific granule, and that this was strongly acidophil in its arlier phase, staining then intensely with phloxin and azocarmine, and less cidophil in its later stage, tending to stain then with orange G and losing to me extent its affinity for phloxin. Precisely the same is now seen to be true f the snake, and, in particular, the loss of affinity for phloxin is clearly indiated by the fact that the stain reveals fewer 'B' granules than does acid fuchsin . 288). A more detailed comparison must await completion of the study of he secretory cycle, but in the meantime it can be said that the degree of greement already reached is an encouraging indication that this approach is kely, as was originally hoped, to make it possible eventually to present a unied account of the comparative cytology of this tissue.

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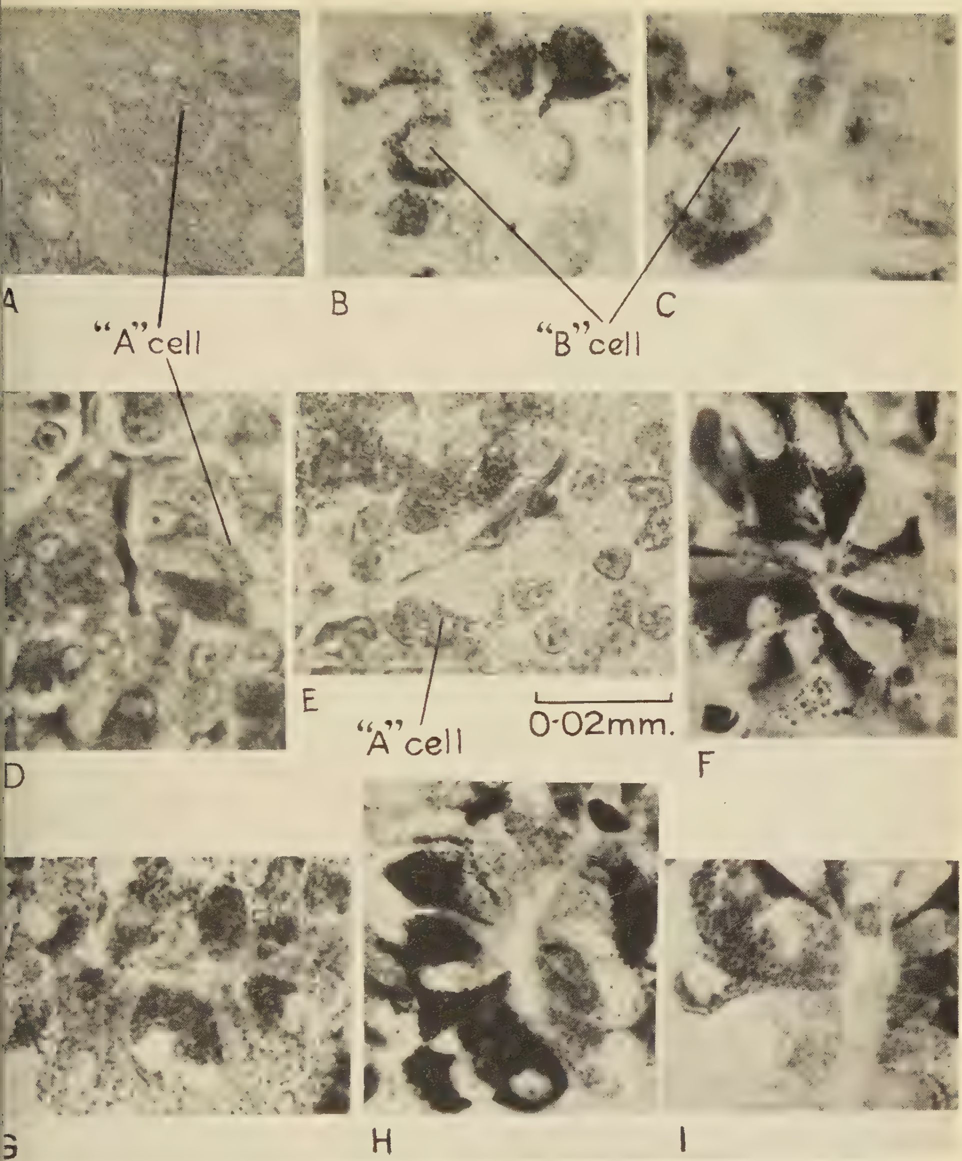

Fig. 1
E. J. W. BARRINGTON

The Stomatogastric Nervous System of *Arenicola*

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With one plate (fig. 3)

SUMMARY

The stomatogastric system of *Arenicola marina* L. is in the form of a plexus of nerve-bundles connecting the supra-oesophageal ganglia and the circum-oesophageal commissures with a ring ganglion situated in the gut wall at the anterior limit of the oesophagus; the plexus continues down the length of the oesophagus. Numerous sense cells occur in the epithelium of the proboscis and oesophagus. The anatomy of the stomatogastric system is essentially the same in *A. ecaudata* Johnston and in *A. claparedii* Levinsen.

INTRODUCTION

THE Polychaete stomatogastric system consists of one or more pairs of nerves connecting the central nervous system of the head region with ganglionic masses in the wall of the gut; the arrangement varies considerably in different species. For instance, in *Eunice* the pharynx and oesophagus are supplied by two pairs of nerves arising from the supra-oesophageal ganglia, and there are several ganglia associated with the gut wall (Heider, 1925). In *Hermione* (Bernert, 1926) a single pair of nerves from the supra-oesophageal ganglia passes forwards, then back along the sides of the oesophagus, then dorsally, to form three pairs of ganglia and a nerve ring, from which arise small nerves supplying the oesophageal wall. In *Petta* (Nilsson, 1911) the stomatogastric nerves arise from the circum-oesophageal commissures and form a small ganglion on the ventral side of the oesophagus, whence pass nerves anastomosing in the wall of the gut. In *Serpula* (Johansson, 1927) the nerves to the oesophagus are associated with those supplying the tentacular crown. In *Glycera* Gravier (1898) described a pair of nerves arising from the supra-oesophageal ganglia, leading to a nerve ring encircling the anterior end of the buccal tube, from which arise eighteen nerves passing back in the wall of the buccal tube to its junction with the pharynx, where are the teeth, a ring of sensory epithelium, and a ganglionic ring with four enlargements; from these, four nerves pass back to the oesophagus, each with two ganglionic swellings. This system had previously been described in part by Jourdan (1891).

In *Arenicola* Ashworth (1904, also Ashworth, 1903, and Gamble and Ashworth, 1900) mentioned that nerves pass into the buccal mass from the anterior lobes of the supra-oesophageal ganglia and from the commissures; he suggested that these might form part of, or be connected with, the stomatogastric system, which he did not describe. More accurate information is

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desirable in view of the physiological interest of this region (Wells, 1937a 1950).

The anatomy of the proboscis apparatus of *Arenicola* was revised by Well (1952). In his nomenclature, the parts of the proboscis are, passing away from the mouth, the buccal mass, pharynx, and post-pharynx, all of which ar eversible. The oesophagus is divided into several regions which need not b distinguished here. Between the gut and the body wall extend the retracto sheath, the gular membrane (or first diaphragm), and the second and thir diaphragms. The gut wall itself contains circular and longitudinal muscl layers.

MATERIAL

The material used consisted of sections of the anterior segments of *Arenicola marina*, and vitally stained preparations of the proboscis and oesophagea walls. I am indebted to Dr. G. P. Wells for the loan of sections stained wit haematoxylin and eosin, and with Hansen's trioxyhaematein and Mallory' phosphomolybdic acid/aniline blue/orange G mixture. Other sections wer impregnated by Holmes's silver method (Holmes, 1943), but this offered n particular advantages and on the whole both fibres and nerve-cell bodie showed best in the Hansen's sections. The nerves of the oesophageal wal may be stained supra-vitally with methylene blue, provided that sufficien time is allowed, for the presence of a sheath round the nerve-bundles prevent rapid penetration of the stain. The most successful method used was t anaesthetize the worms in 7·5 per cent. magnesium chloride, to dissect out th oesophagus, split it open, and to pin it inner side down on plates of wax, wit hedgehog spines (the method used by Alexandrowicz, 1951, for Crustacea) The wax plates were then inverted on sea-water in a shallow Petri dish to whic had been added about a millilitre of Rongalit methylene blue. It was necessar to leave them at least 3 hours to obtain any extensive staining, and they wer sometimes left overnight. The staining was made permanent by treatmen with 8 per cent. ammonium molybdate for at least 3 hours. The wall of th oesophagus was either mounted entire, only the superficial blood-vessels being dissected away, or the muscle layers were separated from the epithelium which was then mounted alone, for the nervous layer was found to adhere t the epithelium and not to the muscles. The preparations were flattened o slides, dehydrated rapidly in ethyl alcohol, cleared in xylene, and mounted i Canada balsam.

THE STOMATOGASTRIC SYSTEM

As Ashworth described, a number of nerves pass to the buccal mass fro the supra-oesophageal ganglia and from the circum-oesophageal commissures There are usually three large nerve-bundles from the anterior part of eac supra-oesophageal ganglion, which enter the dorsal part of the buccal mass These nerves arise close together and immediately form anastomoses; ther may be one or two small bundles in addition. Other nerve-bundles arise a intervals from the anterior borders of the circum-oesophageal commissures

ey vary in size and they also may form anastomoses very near their origins;
ey pass through the muscles of the body wall and forward beneath the
ithelium of the buccal mass, those from the upper half of the commissures
erally, and those from the lower half ventrally. The median ventral bundles
ve the commissures a short distance anterior to the point of bifurcation
the ventral nerve-cord. In one series about seventeen nerves were seen to
ve the commissure on one side, but there does not seem to be a fixed pattern,
even the two sides of the same worm were not absolutely symmetrical in the
e and course of the bundles. All the nerves in the buccal mass branch and
astomose with one another so as to form a plexus of nerve-bundles, which
ntinues into the pharynx; they may be seen in section among the muscles
d between the muscle layers and the epithelium, as in fig. 1, A, where all the
rves shown ventral to the commissures are part of the stomatogastric sys-
n. These proboscideal nerves run back and join a ring ganglion surrounding
gut at the anterior limit of the oesophagus, immediately behind the post-
arynx. As the post-pharynx marks the limit of extrusion, when the pro-
scis is fully everted the ring ganglion is found just within the opening of the
ophagus. Fig. 1, B shows the position of the ring ganglion, round the wide,
ooth, most anterior part of the oesophagus. The main proboscideal nerves
n between the epithelium and the muscle layers, but branches leave them to
ss into the muscle layers, where they anastomose with one another and with
rve-bundles which rejoin the main plexus; hence fibres might pass into
muscle layers from either direction. The retractor sheath receives similar
ndles at the point where it meets the gut, at the junction of buccal mass
d pharynx. Sensory fibres from the sense cells of the epithelium also enter
proboscideal nerves. The whole system is plexiform and connects with the
ophageal ring ganglion, so that it may be called stomatogastric even though
ontains somatic fibres.

Posteriorly, the ring ganglion gives rise to numerous nerve-bundles, for the
st part smaller than those of the proboscis, which pass back as a plexus in
oesophageal wall; they may be seen in figs. 2, A and 3, A, which are from
thylene blue preparations of the stretched oesophageal wall: fig. 2, B shows
ortion of the plexus at a greater magnification.

Part of the ring ganglion, as seen in a transverse section of the oesophagus, is
n in fig. 3, B; nerve-cell bodies, as is normal in annelid ganglia, lie near the
er surface of the ring, particularly near the points of entry of the probos-
eal and oesophageal nerves. Other nuclei are situated on the epithelial side
the ring; these are probably associated with the sensory cells of the epithe-
m, or may be the nuclear portions of those cells. Fibres run round the ring,
d also radially within its substance; these fibres presumably arise from the
erficial cell bodies. In addition, the ganglion is penetrated by the longi-
inal fibres of the proboscideal and oesophageal nerves, which pass between
outer cell bodies and between the radial bundles. It is possible that
ne fibres pass through the ganglion without forming synapses, but the
jority appear not to do so, for the size of the longitudinal bundles

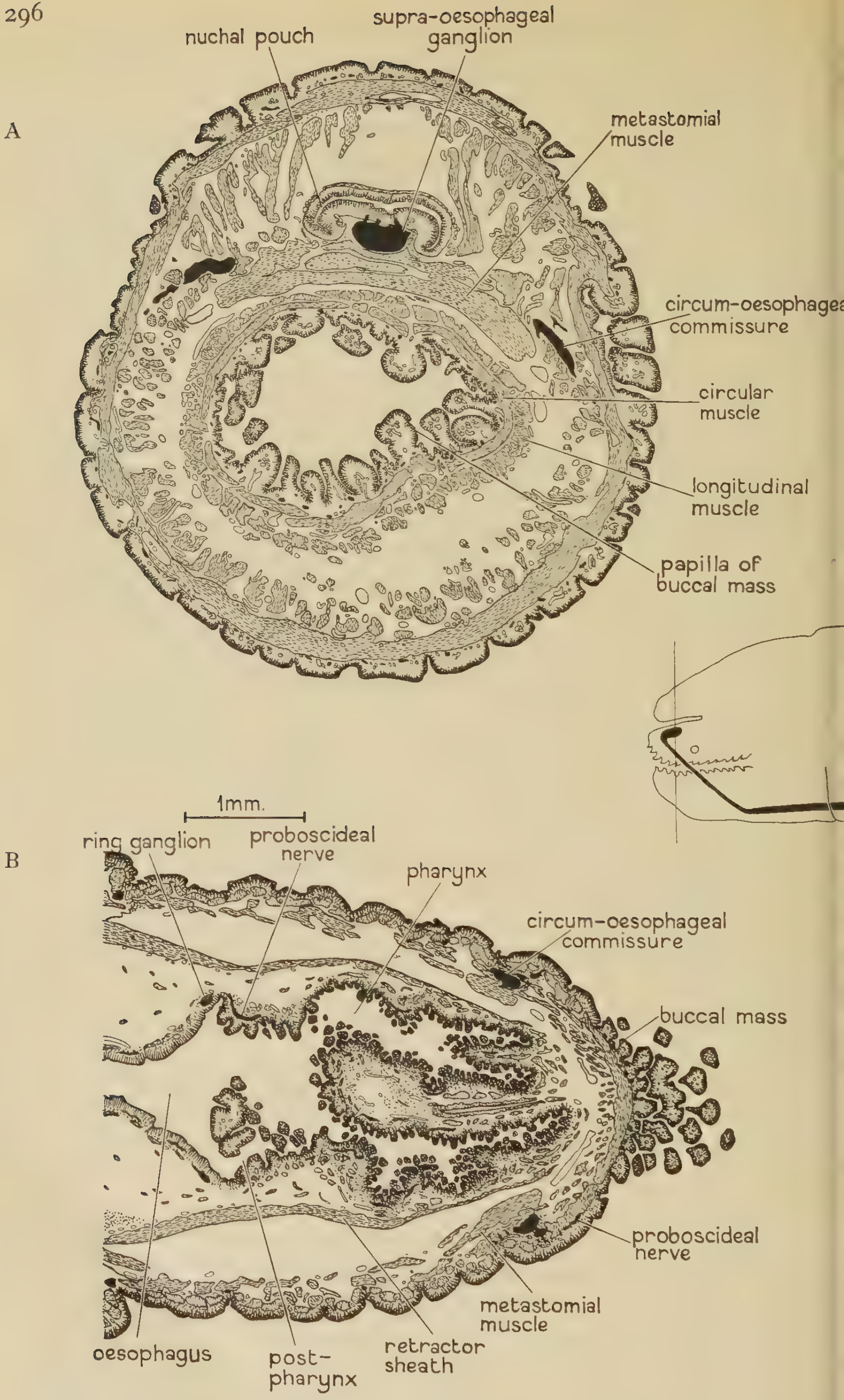

FIG. 1. *Arenicola marina.* Camera lucida drawings. Nerves shown in black. A, transver section in the plane indicated in the inset. B, horizontal section showing the position of t ring ganglion.

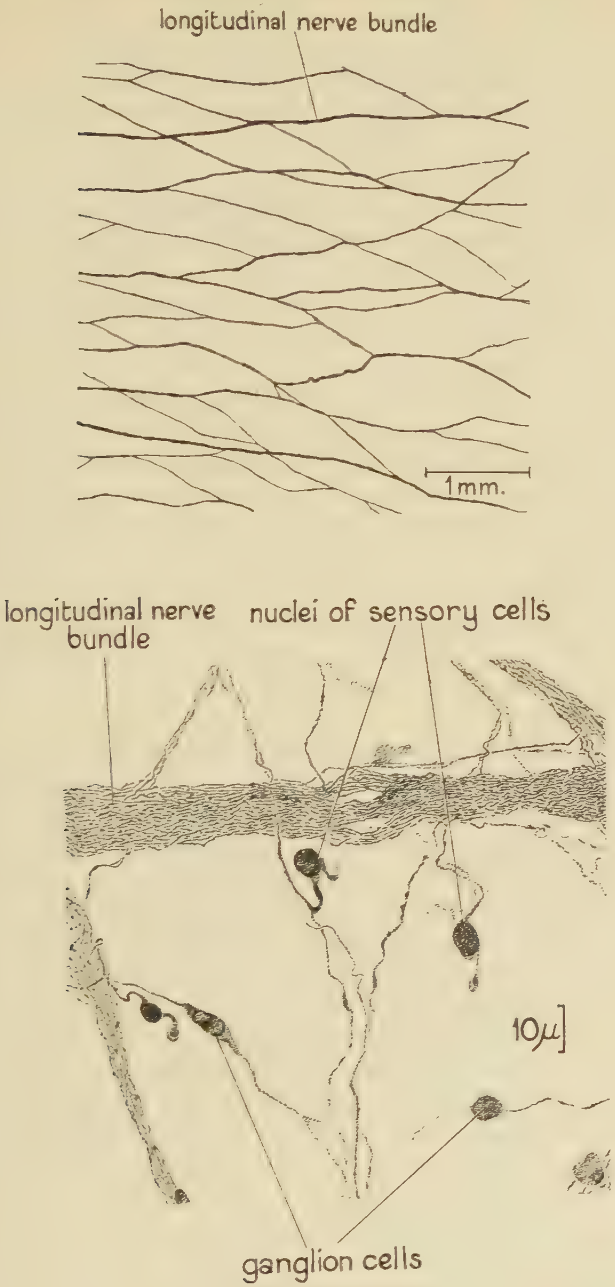

Fig. 2. *Arenicola marina.* Camera lucida drawings of stretched oesophageal wall with uscle layers removed. Methylene blue. A, low-power view of the plexus of the nervous yer. B, part of the plexus at a higher magnification showing nerve-bundles, ganglion cells, d the nuclei of sensory cells. One-twelfth inch oil immersion objective, ×6 eyepiece.

decreases markedly as they enter the ganglion, from either direction. Nerv cell bodies occur in the nerve-bundles of the proboscis and oesophagu especially near the ring ganglion, but are also scattered throughout the lengths, and, at least in the oesophagus, between the nerve-bundles. Groups nerve-cells are found associated with the roots of the proboscis nerves runnin from the anterior side of the commissures; these are, however, less distin than the groups of cells on the posterior borders of the commissures, belo the junctions of the otocyst nerves, which apparently correspond to the later ventral cells of the ventral nerve-cord and are associated with the origins nerves passing to the adjacent muscles such as the metastomial muscles. Th ganglion cells of the nervous layer of the oesophagus are usually bipolar, bu sometimes tripolar; they may be seen in figs. 2, B and 4, B.

The epithelium of the proboscis contains numerous sensory cells of th typical annelidan form, as described by Jourdan (1891, 1892), Retzius (1892*b* Lewis (1897), and Wallengren (1902); they are arranged radially in the papilla of the buccal mass and pharynx, and also occur in the epithelium throughou the length of the oesophagus. The nuclei of the sensory cells lie at the base o the epithelium, and an internal process runs to the nervous layer, usuall bifurcating, the branches joining bundles of the nerve plexus. The interior o the oesophagus is corrugated, so that when it is flattened on a slide split appear, where the sensory cells may be viewed in profile, as in fig. 4, A and B they are shown in perspective in fig. 2, B, and are the darkly stained bodies i the background of fig. 3, A.

Fibres from the nervous layer pass into the circular and longitudinal muscl layers of the oesophagus; in favourable positions they can be seen to end o the muscle-fibres, sometimes applied to the surface of the fibre for a short distance without any apparent modification, sometimes ending in a swollen foo (fig. 5, A and B). Unfortunately, as the tissue must be left in the stain for s many hours no reliance can be placed on the final form of such delicate structures, whose appearance may be an artifact; the actual fibres in the case illustrated (which are both from the same preparation) do not appear to b distorted. The fibres were apparently unbranched, each supplying a singl muscle-fibre. The nerve-fibres of the oesophageal plexus generally were nc seen to branch except in the vicinity of the cell bodies. Retzius (1892*a*) show annelid motor nerves branching on muscle, but does not show details of th actual connexions. Chen (1944) mentions that a gold chloride method wi demonstrate nerve endings on muscle-fibres of the pharynx of *Lumbricus*, bu does not describe them.

Figs. 2, 3, A, 4, and 5 have been taken from the region of the oesophagu between the gular membrane and the second diaphragm: the conditions in th posterior portions of the oesophagus are similar except that the longitudin

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FIG. 3 (plate). A, methylene blue preparation of stretched oesophageal wall, with musc layers removed, showing the plexus of the nervous layer and sensory cells in the epitheliun

B, part of the ring ganglion seen in a transverse section of the oesophagus. Hansen's stai 10 μ.

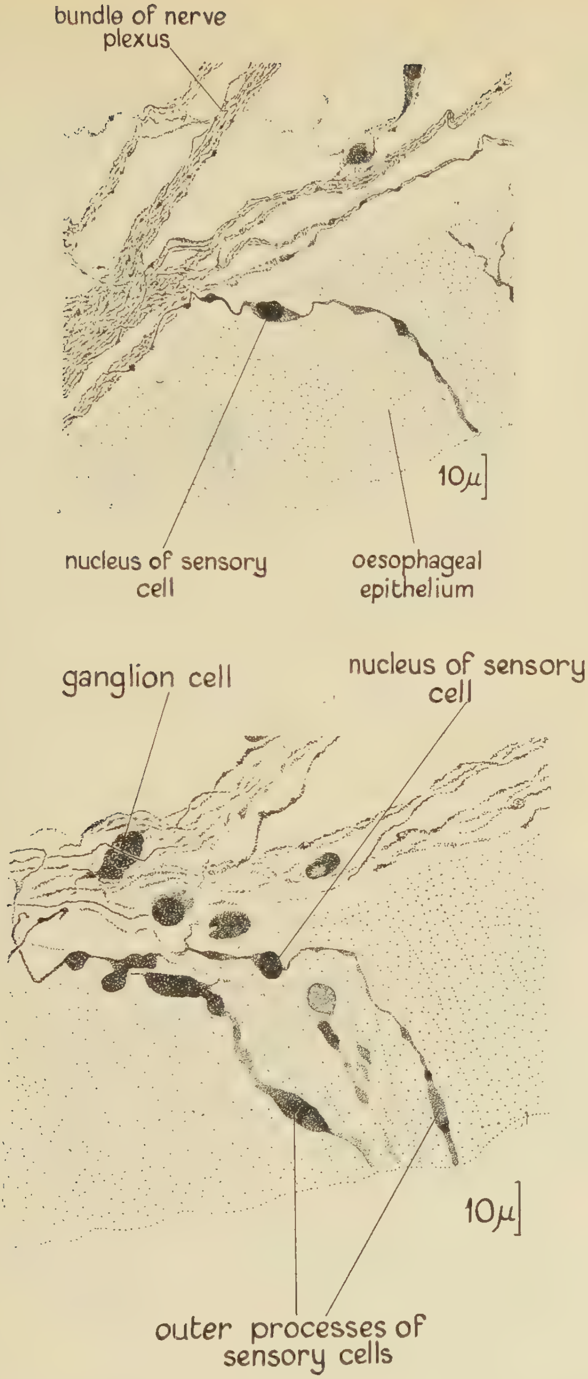


FIG. 4. *Arenicola marina.* Camera lucida drawings of stretched oesophageal wall with scle layers removed. Methylene blue. The sensory cells in the epithelium are seen in file. One-twelfth inch oil immersion objective, × 6 eyepiece.

bundles become smaller further back; the nervous layer as a whole is quite
rich in the most posterior, glandular, portion as in the anterior parts of th
oesophagus. No attempt was made to trace the nerves on to the stomach.

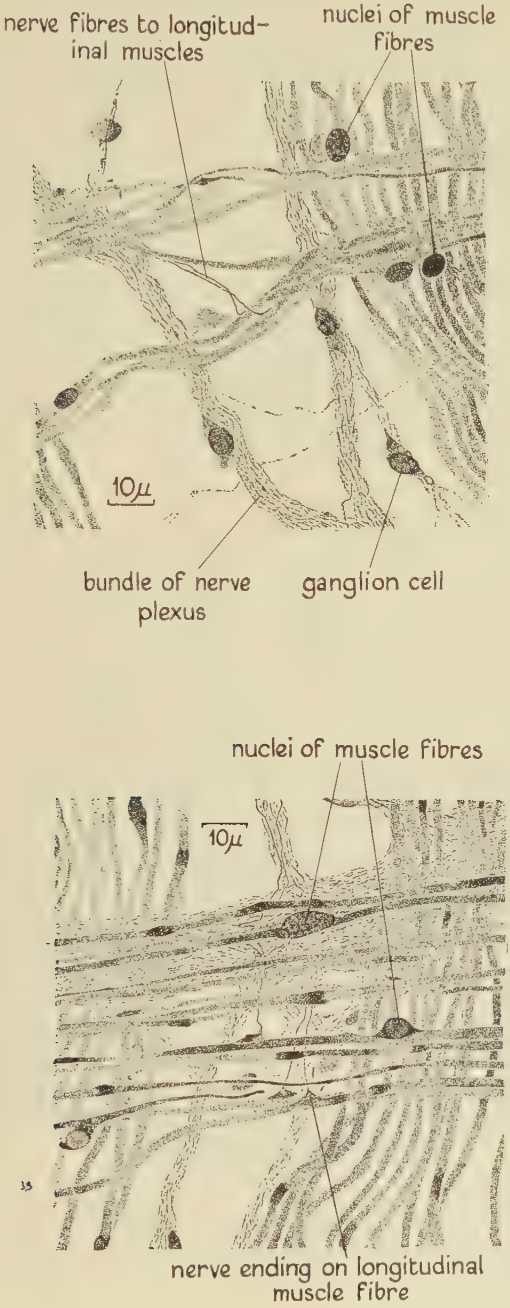
Examination of sections of *Arenicola ecaudata* and *A. claparedii* showed th
the arrangement of the stomatogastric system in these species is essentially th
same as that in *A. marina*. The ring ganglion is situated at the junction of th
post-pharynx and oesophagus, instead of immediately behind the junction
in *A. marina*.

Discussion

The experiments of Wells (1937*a*) on *A. marina* showed that that part of th
gut anterior to the post-pharynx differs physiologically from that behind: th
latter, isolated, exhibits a characteristic intermittent rhythm of contraction
and is excited by adrenalin, while the former, isolated, exhibits continuou
activity and is inhibited by the concentration of adrenalin which stimulat
the oesophagus. When the proboscis is in continuity with the oesophagus th
intermittent rhythm invades the proboscis and may also invade the anteri
three segments of the body wall. The body wall, in its activity and reactions
adrenalin, resembles the proboscis, though there are complications due
differing reactions of the circular and longitudinal muscles (Wu, 1939). Th
excitation of the body wall by the oesophageal rhythm will not extend past
point at which the ventral nerve-cord is cut.

The proboscideal nerves must contain both fibres originating in the centr
nervous system (or in the ganglion cells of the proboscis wall itself) which i
nervate the muscles of the proboscis wall, and also fibres conducting forwar
from the visceral system of the oesophagus and making connexions with th
somatic centres, since the activity of the latter can be modified. It has bee
mentioned that fibres could also reach the muscles of the proboscis direct
from the region of the ring ganglion, through the subsidiary plexus of nerv
bundles which lies in the muscle layers, and connects with the main plexus
branches running both anteriorly and posteriorly. Presumably the probo
cideal nerves also contain fibres running from the central nervous system
the visceral system, and others which supply the retractor sheath, which
innervated at least in part by branches from the proboscideal nerves, thoug
other nerves from the ventral nerve-cord might enter it at its insertion on th
body wall.

Wells states that the boundary between the two physiological regions is
the level of the post-pharynx, whereas the ring ganglion (in *A. marina*) is ju
behind this. If the intermittent rhythm of the oesophagus is assumed
originate in the visceral ganglion cells, some of the numerous nerve-cells
the bases of the proboscideal nerves, immediately in front of the ring ganglio
must also be visceral, so that in *A. marina* the ganglion does not mark t
extreme anterior border of the visceral system. The presence of the ri
ganglion is not essential for the maintenance of the intermittent rhythm, for
the isolated oesophagus is divided behind the ganglion both portions exhib



IG. 5. *Arenicola marina.* Camera lucida drawings of stretched oesophageal wall. Methy-
blue. Nerve-fibres rising from the nervous layer to end on longitudinal muscle-fibres.
-twelfth inch oil immersion objective, ×6 eyepiece.

the characteristic movements; the ring ganglion may then be regarded as specialized part of the oesophageal plexus.

In *Glycera* Jourdan (1891) states that the nerve ring is associated with pad of epithelium especially rich in sensory cells, but this was not noticeab in *Arenicola*; there are numbers of sensory cells throughout the proboscis an oesophagus; in any case the arrangement of the anterior part of the gut in th two animals is not strictly comparable (see Wells, 1937*b*).

The gut of the earthworm, *Lumbricus*, is innervated by both intrinsic an extrinsic autonomic nerves, the latter reaching the gut from the ventral nerv cord by way of the septa (see Millott, 1943, who refers to the earlier litera ture). It is not known if the polychaete gut also receives an autonomi innervation in addition to the stomatogastric; the question would be bes investigated physiologically in the first place.

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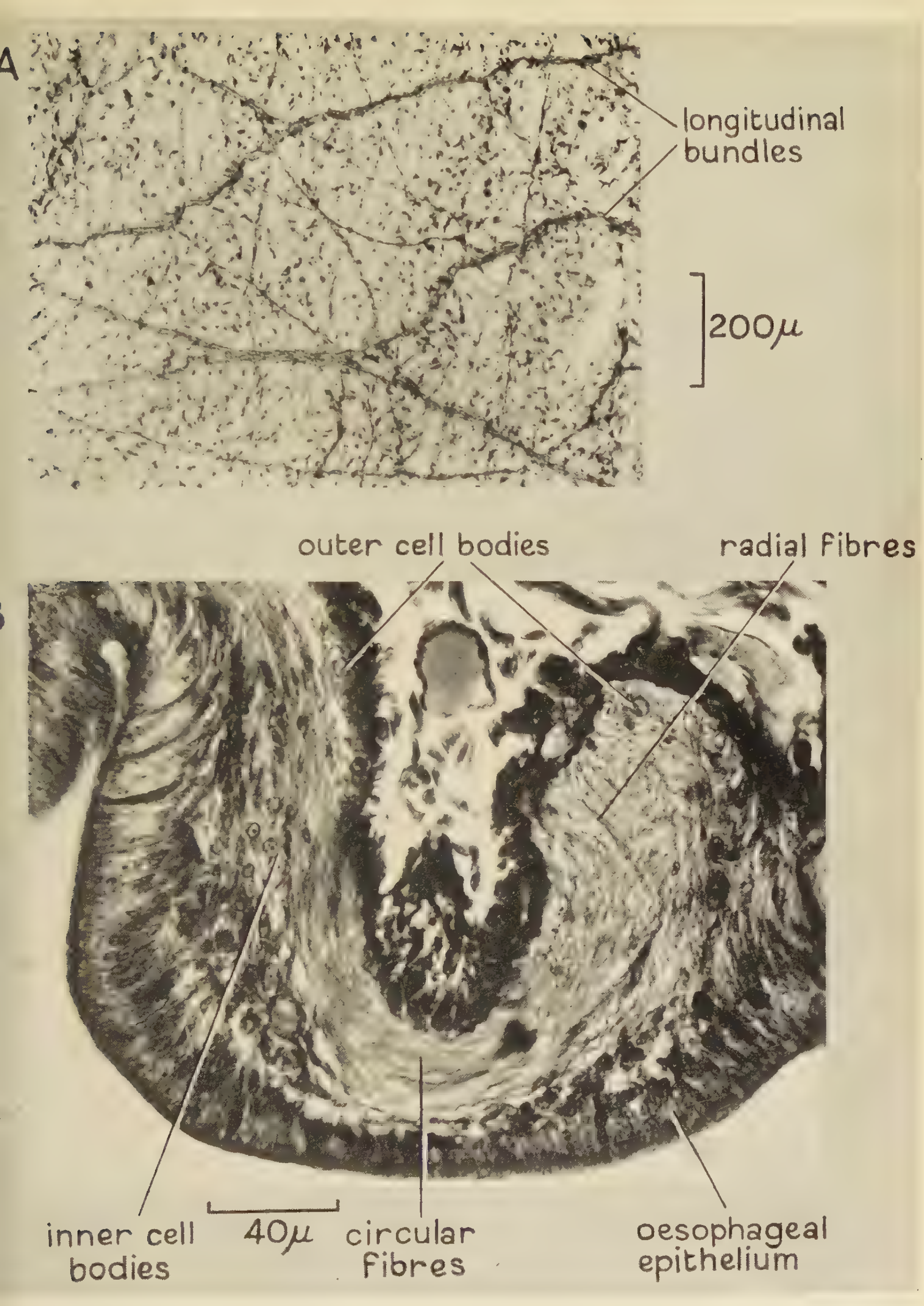

FIG. 3

M. WHITEAR

Observations on Substances that react weakly to the Periodic Acid/Schiff Test

By ARTHUR J. HALE

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With one plate (fig. 1)

SUMMARY

It has been shown that certain mucinous substances which are weakly positive with periodic acid/Schiff technique stain more strongly after treating them with a ution of sodium hydroxide.

The deepening of the staining reaction is produced by making available, for aldehyde duction by periodic acid, more $.CHOH.CHOH.$, $.CHOH.CHNH_2.$, or $.CHOH$ HNHR. groups. This is done without affecting those sulphate or metaphosphate ups responsible for metachromasia.

INTRODUCTION

N histological sections there are present a number of substances loosely described as being of a mucinous nature. Examples of these are the ground stance of cartilage and of other less dense connective tissues, sarcolemma, sement membranes, colloid of endocrine glands, and the mucus of epithelia. stochemically they are related and the basis of the relationship is the sence of a carbohydrate found either as a separate entity or in combination h protein or lipide. The biochemistry of these substances is as yet incom- tely understood, but there are available histochemical methods which give ne indication of the composition of any one member of this family. One these is the periodic acid/Schiff technique (McManus, 1946). Some of the stances mentioned above will give a strong colour reaction with this thod, others will only give a weak reaction. Glycogen, the ground substance cartilage, and certain types of mucus are examples of the former; other es of mucus and sarcolemma are examples of the latter.

The periodic acid/Schiff (P.A.S.) technique (Lillie, 1947; Hotchkiss, 1948; Manus, 1948) is really a Malaprade reaction (Lillie, 1950). It identifies HOH.CHOH., $.CHOH.CHNH_2.$, and $.CHOH.CHNHR.$ groups in ysaccharides, mucopolysaccharides, mucoproteins, glycoproteins, and colipides by production of aldehydes, through periodic acid oxidation, ich colourize the Schiff aldehyde-reagent.

Hale (1946), Gersh (1949), and Roberts and Jarrett (1950) have presented thods which may be of use in differentiating between substances which e a positive reaction with P.A.S. Hempelmann (1940), Friedenwald (1947),

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and Grishman (1948) have introduced methods for differentiating tiss constituents of this type which stain metachromatically. Dempsey and h associates (1946, 1947, and 1950) and Pearse (1949 and 1950) have used metho for identifying different mucopolysaccharides by their degree of basiphilia.

This report is the result of the finding that pre-treatment of histologic sections with a weak solution of sodium hydroxide accentuates, in some case the depth of staining-reaction obtained with the P.A.S. method.

Materials and Methods

As interpretation of the results depended on intensity of the colour r action which developed, care was taken to subject all sections to identic procedures.

The periodic acid solution was prepared as described by Lillie (1947). Th Schiff solution was prepared as suggested by Lillie (1951*a*) and modified t conform with the findings of Longley (1952) and Atkinson (1952) in order t make the results more easily reproducible.

The sulphite rinse between periodic acid oxidation and immersion i Schiff's solution has been shown by Lillie (1951*b*) to reduce the intensity of th staining reaction with periodic acid, and this step has been omitted througl out in order to maintain the strength of any possible positive reaction.

Acetylation blocks .CHOH.CHOH., CHOH.CHNH$_2$., and .CHOH CHNHR. groups (Gersh, 1949; McManus and Cason, 1950) and preven the production of aldehydes by subsequent periodic acid oxidation. D acetylation returns the groups so blocked to their original potential reactivit Acetylation of sections was carried out as described by Lillie (1951*b*). It wa found that deacetylation in 75 per cent. ethanol and ammonia was mo efficient than that in absolute ethanol and ammonia as used by Lillie.

Immersion of sections in hydroxylamine hydrochloride (Danielli, 1949 to block all aldehydes, was used to demonstrate the specificity of the Schiff solution and to identify the nature of the reaction product.

The method for the Feulgen reaction, in so far as it could be carried out paraffin sections, followed the instructions of Danielli (1949).

In all cases, batches of sections containing one slide from each tissue we carried through each procedure. Batches were placed in glass racks and tim were accurately controlled. All sections, irrespective of the fixative used, we taken through iodine and thiosulphate when being brought down to wat before staining. All solutions were renewed from standard stock bottles ea day. Washing was carried out in running tap water. Glasgow tap water is ve pure and constant in quality (Garven and Gairns, 1952) and no harm appea to be caused by washing sections in it. Before and after the periodic acid sol tion the sections were washed in 70 per cent. ethanol. All sections we dehydrated in 90 per cent. ethanol and two changes of absolute ethan cleared in xylene, and mounted in D.P.X. Counterstains were not used.

The tissues used were known to give a weakly positive P.A.S. reaction in least part of their substance. They were:

Human tissues *fixed in 10 per cent. neutral formalin*: heart (lipofuscin), cervix, prostate, rectum, pancreas, trachea, submaxillary gland;
fixed in mercuric formalin: rectum, amyloid spleen, aorta, umbilical cord, amyloid liver, myxoma, heart valve;
fixed in Bouin's fluid: lung (pneumonic exudate), umbilical cord, small intestine, stomach.

Rabbit tissues *fixed in 10 per cent. neutral formalin*: duodenum, kidney;
fixed in Bouin's fluid: bone marrow.

Rat tissues *fixed in 10 per cent. neutral formalin*: eye, mesentery.

Representative batches of sections were carried through by each of the llowing procedures after having been brought to water. Room temperature pproximately 20° C.) was used except where otherwise stated.

(1) Periodic acid 15 min.; wash; Schiff soln. 30 min.; wash.
(2) Schiff soln. 30 min.; wash.
(3) $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; periodic acid 15 min.; wash;
chiff soln. 30 min.; wash.
(4) $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; Schiff soln. 30 min.; wash.
(5) Acetylation; wash; periodic acid 15 min.; wash; Schiff soln. 30 min.;
ash.
(6) Acetylation; wash; deacetylation; wash; periodic acid 15 min.; wash;
chiff soln. 30 min.; wash.
(7) $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; acetylation; wash; periodic acid
min.; wash; Schiff soln. 30 min.; wash.
(8) Acetylation; wash; $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; periodic acid 15
in.; wash; Schiff soln. 30 min.; wash.
(9) Deacetylation 15 min.; wash; periodic acid 15 min.; wash; Schiff soln.
min.; wash.
(10) Deacetylation 24 hrs.; wash; periodic acid 15 min.; wash; Schiff soln.
min.; wash.
(11) Diastase 30 min.; wash; periodic acid 15 min.; wash; Schiff soln. 30
in.; wash.
(12) 0·5 per cent. toluidin blue 30 min.; wash.
(13) $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; 0·5 per cent. toluidin blue 30 min.
(14) $0{\cdot}2\overline{N}$ NaOH 15 min. at 22° C.; wash; periodic acid 15 min.; wash;
droxylamine 60 min.; wash; Schiff soln. 30 min.; wash.
(15) Periodic acid 15 min.; wash; hydroxylamine 60 min.; wash; Schiff
ln. 30 min.; wash.
(16) Periodic acid 15 min.; wash; hydroxylamine 24 hrs.; wash; Schiff
ln. 30 min.; wash.
(17) Hydroxylamine 1 hr.; wash; $0{\cdot}1\overline{N}$ HCl. 15 min.; wash; Schiff soln.
min.; wash.
(18) Periodic acid 15 min.; wash; Schiff soln. 30 min.; wash in three changes
sulphite soln., 5 min. in each change.

(19) 0·2$\overline{\text{N}}$ NaOH 15 min. at 22° C.; wash; periodic acid 15 min.; was Schiff soln. 30 min.; wash in three changes of sulphite soln., 5 min. in each

SIGNIFICANCE OF METHODS AND RESULTS

(1) This is the standard technique used to identify .CHOH.CHOH .CHOH.CHNH$_2$., and .CHOH.CHNHR. groups by production of ald hydes by periodic acid oxidation and identification of the aldehydes by r colourization of Schiff's solution.

(2) Immersion directly in Schiff's solution, which gives no reaction, show that none of the colour produced in (1) is caused by the presence of 'fre aldehydes in the sections or by spontaneous recolouration of the Schiff solution.

(3) This is the method which increases the intensity of the staining reactio of certain substances which otherwise give only a pale colour with procedure (1 The maximum amount of change from the standard appearance occurred i the first few minutes of incubation of sections in 0·2$\overline{\text{N}}$ NaOH at 22° C. Time or temperatures in excess of this produced no further change. When compare with the results from (1) the sections of human rectum and rabbit duoden showed the most striking changes. The Brunner's glands of the rabbit duodenum changed from a faint pink colour with the standard technique to deep magenta colour after treatment with the NaOH solution (fig. 1, A and B) The goblet cells of the human rectum changed from a pink to a very dee magenta colour (fig. 1, C and D). The fibrin clot of the pneumonic exudate o the lung increased slightly its depth of colour after treatment. In all the tissue there was a slight increase in intensity of staining of reticulin, certain irregula areas of collagen and sarcolemma, and in the amount of diffuse pink connectiv tissue background.

(4) Incubation of sections in NaOH, without subsequent periodic aci oxidation, followed by immersion on Schiff's solution was done to find out i aldehydes were being produced. No colour was produced.

(5) The acetylation blocks the periodic acid reactive groups. Colour wa not produced.

(6) This is a check on the specificity of (5) by removing the blocking agen and it produced the same colour reaction as (1).

(7) The acetylation after incubation in NaOH was introduced to fin whether the alkali was releasing groups, other than those enumerated above which were subsequently being oxidized to produce aldehydes. No colour wa produced. This showed that any groups released by the NaOH were of th above type.

FIG. 1 (plate). A, rabbit duodenum. P.A.S.
B, rabbit duodenum. NaOH+P.A.S.
C, human rectum. P.A.S.
D, human rectum. NaOH+P.A.S.

The light-source, aperture, and exposure were the same in all four cases. Ilford filte Nos. 104 and 108 were superimposed.

(8) It was possible that the action of the NaOH might only be a deacetyla- on, so this step was introduced. An excellent deacetylation, identical to that oduced by the ethanol-ammonia mixture, was brought about, but in addition, creased staining of rectal goblet cell and Brunner's gland mucus was seen.

(9) As the NaOH can deacetylate this step was carried out to show that the use of the increase in intensity was not of this nature. This procedure, ving the same reaction as (6) and (1), did not produce any increase in inten- y of the staining reactions as enumerated in (3). Therefore the increased lour in (3) is not due to deacetylation.

(10) As in (9).

(11) The diastase digestion was introduced to show that none of the in- eased colour produced in (3) was associated with the presence of glycogen. he glycogen reactions of the squamous epithelium of the cervix and rectum, the walls of the blood-vessels of the umbilical cord, of the macrophages the pneumonic exudate, and of the chondriocytes of trachea, were removed.

(12) This is the standard procedure for producing metachromasia.

(13) It was thus shown that the NaOH did not remove those groups re- onsible for the production of metachromasia, as the appropriate metachro- atic reactions of all tissues used were obtained.

(14) (15), and (16) These procedures were carried out to show that the colouring of the Schiff's solution was due only to the presence of aldehydes, iether procedure (1) or (3) was used. In all three procedures a pale reaction rsisted in what were normally strongly staining areas.

(17) Blocking of 'free' aldehydes and production of a Feulgen reaction were rried out for comparison with the sites of colour production in the pro- dures. A faint positive reaction was produced in the nuclei of the rectal ithelial cells, the retinal cells, and the pancreatic acinar cells. There was o a persistent positive reaction in the cartilage of the trachea and throughout e connective tissues and all nuclei of the rat mesentery.

(18) and (19) Rinsing sections in sulphite solution after leaving the Schiff's agent prevented the formation of non-specific colour formed by atmospheric idation of adsorbed Schiff's reagent. These sections showed no difference compared with those of (1) and (3) respectively.

DISCUSSION

The P.A.S. reaction gives a positive result, with a varying degree of in- isity, with polysaccharides, mucopolysaccharides, mucoproteins, glycopro- ns, and glycolipides, as stated before.

The main representatives in each of these groups, in mammals, are (Meyer, 45):

polysaccharides: glycogen
mucopolysaccharides: *neutral*: blood-group substances
    acid: (*simple*): hyaluronic acid
        (*complex*): mucin, heparin, chondroitin sulphate

mucoproteins: submaxillary mucoid, serum mucoid, seroglycoid
glycoproteins: serum albumin and globulin
glycolipides

Substances which stain metachromatically, at least as far as they are e countered in histological material, are polysaccharides containing uro acids in addition to sulphate groups (Lison, 1935), or polymeric metaph phate groups (Bank and Bungenberg de Jong, 1939; Michaelis and Grani 1945; Michaelis, 1947). The causation of metachromasia is incomplet understood, but it is generally considered to depend on polymerization of t dye molecules in the presence of these chemical groups (Kelly and Mill 1935; Bank and Bungenberg de Jong, 1939; Hempelmann, 1940; Michae and Granick, 1945; Michaelis, 1947; Landsmeer, 1951; Massart and othe 1951). The substances of interest in this investigation are the mucopo saccharides, such as rectal mucus or heparin, and the glycoprotein amylo

Glycogen can be eliminated by diastase treatment. Glycolipides are usually evident in the paraffin sections in the areas studied. This can be co firmed by a chloroform-methanol extraction method (Pearse, 1951). We thus left with the necessity of differentiating between mucopolysaccharid mucoproteins, and glycoproteins.

Davies (1952) states that hyaluronic acid, a simple acid mucopolysacchari considered to be the main constituent of the 'ground substance' in connecti tissues, will only stain very faintly with the P.A.S. technique according to t formula of Meyer and Fellig (1950). He himself found that it neither sta metachromatically nor with periodic acid if it is properly purified. My o findings with completely purified and incompletely purified hyaluronic ac preparations confirm this. With regard to chondroitin sulphuric acid, complex (i.e. sulphate-containing) acid mucopolysaccharide, Davies poi out that according to the formula given by Haworth (1946), the reaction w P.A.S. should be strong; but according to that of Meyer, Odier, and Siegr (1948), the reaction should be very faint.

Lillie (1949) reports that the mucin (? hyaluronic acid) of the connecti tissue of the umbilical cord stains metachromatically but gives no colour re tion with periodic acid. Sylven (1941, 1945, and 1950), Davies (1943), Wislo and others (1947), Campani and Reggiani (1950), Mancini (1950), and Wislo and Sognnaes (1950) report the presence of metachromatic ground substan in granulating tissue and other actively growing connective tissues. Me (1946) states that

> 'young growing fibroblasts secrete hyaluronic acid, which is followed by the sec tion of chondroitin sulphate and of a precursor of collagen, the latter a non-fibro and soluble protein. By local acidification in the immediate neighbourhood of fibroblasts, the precursor is denatured by the polysaccharides, the latter acting anionic detergents rolling up the peptide chains along the acidic groups of the fibro polysaccharide molecules. Most of the hyaluronate is removed enzymatical leaving the more firmly bound chondroitin sulphates as a network on the surface the fibres.'

Thus it appears that the metachromatic ground substance stained by numerous investigators is likely to be free chondroitin sulphate which is present in actively multiplying connective tissue. Thus as hyaluronic acid is P.A.S. negative and not metachromatic it does not come into the initial classification of P.A.S. positive substances.

If the strongly P.A.S. reacting substances are left out in Meyer's classification it is now necessary to differentiate between those mucopolysaccharides which react weakly and the persistently more weakly reacting mucoproteins and glycoproteins.

Chondroitin sulphates, which are bound in collagen to form a mucoprotein, or to globulin as in amyloid to form a glycoprotein (Krakow, 1897; Lillie, 1950), show varying degrees of metachromasia and of reactivity with the P.A.S. method. Free chondroitin sulphate and that bound in cartilage show a strong metachromasia and a strong reaction with P.A.S.

Human rectal mucus and the mucus of Brunner's glands in the rabbit duodenum are consistently strongly metachromatic and weakly positive with periodic acid. Rabbit Brunner's glands are characteristic in this respect (Chapman, 1952). Lillie (1951*b*) has pointed out, in discussing intestinal mucus and its staining reaction with the Bauer (1933) and Casella (1942) techniques, that there appears to be an inverse relationship between strongly P.A.S. positive substances and metachromatically positive ones; for example, gastric mucus stains very strongly with periodic acid but is only very weakly, if at all, metachromatic. Rectal mucus gives the reverse reaction. He suggests that a mucus weakly P.A.S. positive but strongly metachromatic has fewer groups available for aldehyde production with the P.A.S. technique because of a longer chain structure.

Mucoproteins such as sub-maxillary mucoid are consistently weakly P.A.S. positive.

Procedure (3) causes an increase in the P.A.S. staining reaction of human rectal mucus and of the mucus of the rabbit Brunner's glands. The first is a complex acid mucopolysaccharide. The exact nature of the second is not known. An increased depth in staining also occurs in reticulin and on the surface of collagen, both of which are considered to be mucoprotein (Meyer, 1952). The change in reaction occurs partially in the clot of pneumonic exudate, which is probably a mixture of serum mucoprotein and glycoprotein. It does not take place in sub-maxillary mucoid, which is a mucoprotein. It does not take place in amyloid, which is a glycoprotein (Krakow, 1897; Lillie, 1950). Gersh (1949) intimates that 0·00001 $\bar{N}$ sodium hydroxide, acting for one hour at an unspecified temperature, weakens the P.A.S. staining reaction of the 'glycoprotein' of the Golgi apparatus. He does not report the effect on the surrounding tissues.

Meyer and Rapport (1951), in discussing the nature of the bonds between the mucopolysaccharide and the protein in the mucoproteins of connective tissue, intimate that they extract the mucopolysaccharides with a 0·33 $\bar{N}$ solution of NaOH at 0° C. Meyer (1952) suggests that mucoproteins of connective tissue are mucopolysaccharides bound to protein by strong electr valent forces. Therefore the staining reaction change which I have obtaine may be due to the breaking of weaker bonds in certain mucoproteins.

As the change in reaction appears more clearly in mucus, it may be that is occurring more selectively in mucoitin sulphates as distinct from chondroitin sulphates. It is thus a possibility that the variation in reaction ma be due to the different hexosamine present; acetylglucosamine in mucoit sulphates and acetylgalactosamine in chondroitin sulphates. Morgan and Elsc (1934) have shown that alkaline hydrolysis of acetylglucosamine and acety galactosamine (acetylchondrosamine) can be used to give quantitati colorimetric estimations of their products with paradimethylaminoben aldehyde. Aminoff, Morgan, and Watkins (1952) show that by this metho acetylchondrosamine gives only 23 per cent. of the degree of colour reaction acetylglucosamine, and blood group mucoids give only 7–10 per cent. Th exact mode of reaction is not known but it is suggested that the alkali forms a oxazoline or a pyrazine with the hexosamine. It is not known whether thes products are P.A.S. positive or not. It has been stated (Mann, 1902; Dempse and others, 1947) that fixation of tissues in formaldehyde will prevent th colour reaction. Frey-Wyssling (1948) states, in discussing the chemical natur of cytoplasm, that formaldehyde is thought to fix tissues by forming bridge between neighbouring polypeptide chains. So it may be that the difference i the type of hexosamine present might influence the P.A.S. reaction afte alkaline hydrolysis, though the effect of the formalin fixatives must be born in mind.

Mucopolysaccharides, according to Meyer's definition (1945), are polysaccharides which contain hexosamine as one component, whether the occur free or bound to substances of higher molecular weight. Mucoprotein are those substances which contain a mucopolysaccharide in firm chemic union with a peptide, where the hexosamine content is greater than 4 per cen Proteins which contain less than 4 per cent. hexosamine are classified glycoproteins. Thus the selectivity of the change in reaction may be due to th relative amount of hexosamine present.

If all the preceding arguments are reconsidered it appears that the selectivit of the reaction may be due to one or more of the following causes:

(*a*) The number of reacting molecules available, perhaps depending on th chain length of the substance.
(*b*) The degree of chemical union with protein.
(*c*) The type of hexosamine present.
(*d*) The relative amount of hexosamine present.

All that can be said regarding the nature of the extra groups being mad available, by the caustic solution, for aldehyde production by periodic aci is that:

(*a*) No aldehydes are being produced by the alkali as shown by procedu (4), where no colour is produced.

(*b*) The groups which are being made available are .CHOH.CHOH., CHOH.CHNH$_2$., or .CHOH.CHNHR. groups, as shown by proedure (7), in which all colour reaction is blocked by acetylation.

(*c*) The action of the alkali is not just a deacetylation as suggested by proedure (8), in which the full colour reaction is produced, because procedures 9) and (10) restore the normal colour reactions to the tissues but do not intenify the mucus-reactions.

(*d*) The sulphate or metaphosphate groups thought to be responsible for netachromasia are not affected; this is shown by procedure (13), where the netachromasia remains after alkaline treatment.

(*e*) None of the increased colour intensity is due to absorbed leucofuchsin vhich has been subsequently oxidized in the atmosphere, as shown by proedures (18) and (19).

(*f*) An attempt to show by procedures (14), (15), and (16) that the increased olour reaction was due to the eventual production of aldehydes was prevented y the retention of a slight degree of colouration in normally strongly reacting reas in all three procedures. It is known that the condensation of hydroxylmine with aldehydes is dependent to a certain extent on the pH of the reaction; s the pH rises, within certain limits, so does the rate and degree of condensaion (Ölander, 1927). It may be that these areas, which are known to be of cidic nature (Dempsey and others, 1947), prevent complete condensation nd thus leave a certain amount of aldehyde available to recolourize the Schiff solution.

CONCLUSIONS

It has been shown that certain substances that are weakly positive to the eriodic acid/Schiff reaction can be made to stain more strongly by first xposing them to a solution of sodium hydroxide.

It is suggested that this may provide a method of differentiating between veakly staining long chain mucopolysaccharides, mucoproteins, and glycoroteins. The first deepen markedly in colour, the second show an inconstant mall increase in depth of colour, and the last remain unchanged.

The relationship of the strength of the colour reaction to the varying chemical tructure is not definite; the colour may depend on the chain length, the deree of conjugation with protein, or possibly on the type or amount of exosamine present.

From the notes on methods and results it can be shown that the difference n staining reaction is produced by making available more .CHOH.CHOH., CHOH.CHNH$_2$., or .CHOH.CHNHR. groups for aldehyde production y periodic acid without affecting those sulphate or metaphosphate groups vhich give certain tissues their metachromatic affinities. Whether the requisite roups for aldehyde production are made available by protein extraction or by ome other reaction is not yet known. The process does not appear to be a eacetylation.

The investigation is being pursued with a wider range of tissues and other

methods which are being developed. It is hoped to eliminate fixation errors
the use of frozen-dried material.

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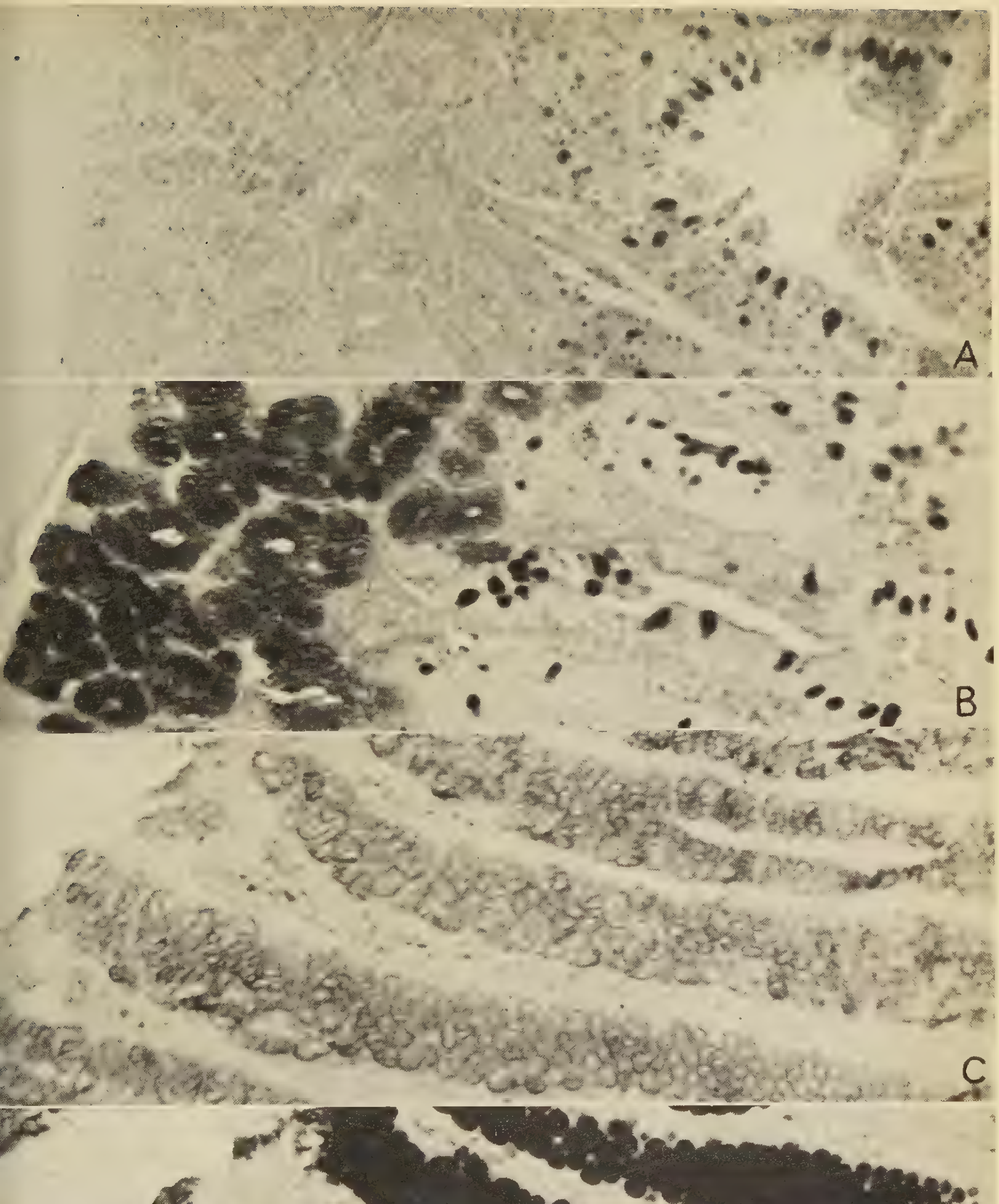

Fig. 1
A. J. HALE

The Lipoid Contents of the Golgi Bodies in the Oocytes of the Indian Water Spider, *Lycosa birmanica*

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Summary

1. The Golgi bodies of the oocyte of *Lycosa birmanica* do not contain triglyceride.
2. They contain phospholipine, lipo-protein, and protein.
3. They are granular and all of approximately equal size. Any variation in their ze or shape is due to the irregular deposition of phospholipine on them.
4. Fine bodies resembling Golgi bodies in appearance and reacting like them with smium tetroxide and silver nitrate consist of phospholipine.
5. The Golgi bodies in the material studied appear not to multiply by division, but o arise in the ground cytoplasm independently of one another. They may be regarded s nutritive bodies.

Introduction

IT is the intention here to study the chemical nature of the Golgi bodies in the oocytes of *Lycosa birmanica* Thor. during the growth period. The Golgi bodies in the oocytes of this animal are of fairly large size and can be een even in the living condition. Observations were made on frozen sections y the use of modern cytological techniques.

Technique

Silver and osmium techniques were employed to identify Golgi bodies. During the breeding season (February to April) fresh ovaries were fixed for 4 hours in the fluids of da Fano, Ramon y Cajal, and Aoyama, and silver npregnation was carried on for 48 hours in 1·5 per cent. silver nitrate soluon. The impregnated silver was reduced and fixed, and the wax sections f this material were toned to remove excess of silver. Material was also reated by Ludford's modification of the Mann-Kopsch osmium technique. ost-osmication was done for 6 days and the wax sections of this material ere bleached with potassium permanganate. The formulae of the fluids in oth the techniques were taken from Bolles Lee's *The microtomist's vade-mecum* 946 edition). The Golgi bodies looked black with these techniques; the itochondrial region appeared as a light-brown patch with the osmium technique. This patch was ignored in the present study.

For the study of lipoids ovaries were fixed in calcium-formaldehyde (Baker, 944) and cut into frozen sections. Triglycerides, phospholipines, and liproteins were identified by selective staining and solubility tests (Krishna,

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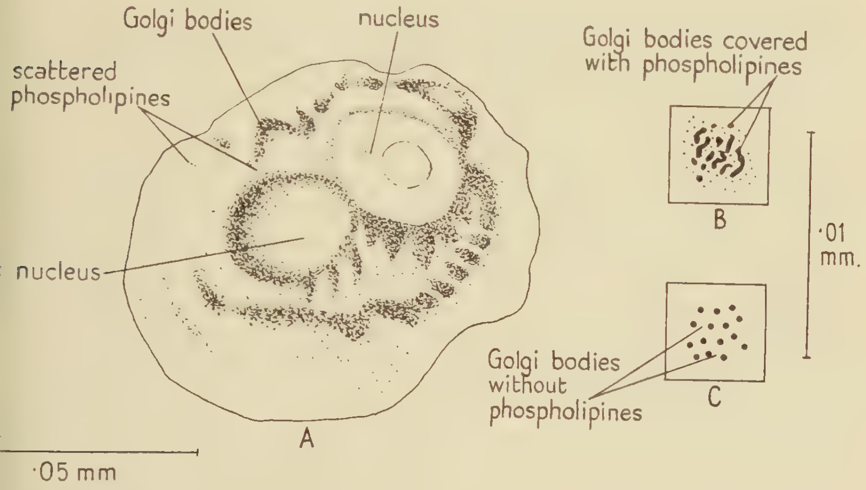
1950). The frozen sections were stained with nile blue sulphate for t glycerides (Cain, 1947*a*, 1948). These were then dissolved in acetone and t phospholipines were stained with sudan black B (Lison, 1936 and 195 Baker, 1944) and acid haematein (Baker, 1946 and 1947; Cain, 1947*b*). Th were also removed with ether and alcohol and the remaining lipoids, the lip proteins, were stained with sudan black B. Sudan IV (Baker, 1944; Gaten and Painter, 1946) was also used for triglycerides and phospholipines as control. The lipo-proteins were stripped of their lipoids in boiling alcoh boiling ether, and finally in frozen ether (McFarlane, 1942). The extracts the sections were always tested for their contents by classical spot tes (Feigl, 1947).

During the course of this study certain tests for proteins were also em ployed. These were standard tests of ninhydrin, aldehyde tests for trypt phane, and Millon's test (Bensley and Gersh, 1933). Proteins were al removed by the enzyme action of pepsin. The frozen sections were firm stuck to the slides over a gelatine film and the slides were incubated wi pepsin solution (0·06 per cent.) in acetic acid medium (Tiselius and Erikso Quensel, 1939) at pH 1·5 (Sahyun, 1944) at 37° C. The enzyme action w complete in 12 hours.

OBSERVATIONS

In young oocytes the Golgi bodies appear near the nucleus and follow t yolk nucleus throughout the growth period. In osmium and silver prepar tions the Golgi bodies appear as small and large granules and also as bodi of irregular shape (fig. 1, A). In living oocytes, too, the irregular bodies an large granules could be seen when the light was to some extent cut off b tilting the mirror of the microscope. These bodies in frozen sections a coloured blue or blue-black with sudan black B (fig. 1, B), and orange wit sudan IV. They are not coloured red by nile blue. These reactions sugge that they do not contain triglycerides.

The intensity of the sudan stains did not appreciably change when t sections were treated with acetone. The Golgi bodies gave a positive reactio with the acid-haematein test, showing in them the presence of phospholipine The sections were then treated with alcohol and ether in succession. Th removed phospholipines from the Golgi bodies and also from other parts the section. The small granules had all dissolved. The irregular bodies lo most of their substance and only circular bodies were left in their place. Th large granules also lost some of their contents and now looked slightly reduce in size, smooth and circular (fig. 1, C). These bodies did not stain wit sudan IV, but took a blue colour with sudan black B. Further treatment wit alcohol and ether did not dissolve any more lipoids. Thus the phospholipin had been removed and the blue colour with sudan black B indicated t presence of lipo-proteins in the Golgi bodies. The sections were then treate with boiling alcohol and boiling ether and then with frozen ether for 12 hour This treatment completely stripped the Golgi bodies of their lipoid content ey could now no longer be coloured by sudan black B, but gave positive ılts with all the protein tests employed here. The removal of the lipoids siderably reduced the size of the Golgi bodies, but their granular shape not change; rather it became more distinct than before. The remnants the Golgi bodies, consisting mainly of protein, then looked like smooth nules and took the protein stains uniformly.


Golgi bodies
nucleus
scattered phospholipines
nucleus
A
·05 mm
Golgi bodies covered with phospholipines
B
·01 mm.
Golgi bodies without phospholipines
C

I. A, section of an oocyte fixed in Ludford's modification of Mann-Kopsch's fluid. ched. B, a part of a frozen section of an oocyte fixed in calcium-formaldehyde and coloured udan black B. C, the same as B after treating the section with alcohol and ether in succession.

'hese sections were incubated with pepsin solution at 37° C. and the ction was watched every hour for 12 hours, when the sections almost ppeared in the enzyme solution. For about 3 hours there was no apprele action. After that the signs of disintegration became distinct. The ains of the Golgi bodies were the first to be attacked by the enzyme, spectively of their position in the section. First they became thin in the tre and after an hour or so looked hollow. The process of thinning proled towards the periphery, and in about 6 hours, when the general cytom began to disintegrate, the remains of the Golgi bodies were visible by the thin circular outline that marked their margin. This outline gave itive results with protein tests and was seen till the last remains of the plasm dissolved. The pepsin solution apparently had little action on this ine, although the surrounding cytoplasm was rapidly dissolving. Disgration of the general cytoplasm also began in the centre of the oocyte proceeded radially towards the periphery. In the end there remained a very thin outline marking the margin of the section. This did not take stain and did not show any structure even when it was seen through an mmersion objective. The number of the Golgi bodies varied with the

size of the oocyte. They were few in young oocytes and many in the old on
There was no evidence to suggest any relation among the granules at
stage of the oocyte growth. On the other hand, they appeared to be form
in the cytoplasm independently of one another. When the phospholipine
been removed, they all appeared to be of the same size.

Discussion

The observations lead to the conclusion that the Golgi bodies in *Lyc*
birmanica are granular, mainly consisting of proteins and lipo-protei
Covering the granule there is a membraneous structure of a doubtful chem
nature—probably proteins, different from those in other parts of the granu
On these granules phospholipines are deposited. Sometimes the dep
covers more than one granule lying near each other, and the Golgi bod
under a common cover of phospholipines, appear in various shapes and si
When the phospholipines are removed the granules become distinct and l
separate from each other. What looked like fine Golgi bodies in the sil
and osmium techniques were really scattered phospholipines and all of th
dissolved in alcohol and ether without leaving any trace behind. Only
large granules showed the usual structure of the Golgi bodies. The li
protein and the protein contents of the Golgi bodies could be seen only wh
the phospholipines had been removed. The enzyme action by its peculiar
in first attacking the centre of the Golgi granule showed that the proteins
the centre were different from those on its periphery. It was interesting
observe that after the removal of the phospholipines all the Golgi granu
were of the same size and shape. Their outward difference was due to
irregular deposit of phospholipines.

There was no evidence to show the protoplasmic nature of the Golgi bod
or their multiplication by division. On the contrary, the observations sugg
that they are separate objects which originate in the cytoplasm and h
a nutritive function.

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ytological Studies of the Neurones of *Locusta migratoria*

Part I. Cytoplasmic Inclusions of the Motor Neurones of the Adult

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With one plate (fig. 2)

Summary

The motor neurones of *Locusta migratoria* have the following inclusions in their oplasm:

(*a*) granular and filamentous mitochondria;
(*b*) lipochondria of various sizes. These are osmiophil and sudanophil. They also gregate neutral-red and other vital dyes. These 'neutral-red bodies' are shown not to new formations in the cells, as was believed by Beams and King. The lipochondria y perhaps be homogeneous bodies, though in fixed preparations and also in life y sometimes appear to have a cortex and medulla. The osmiophil platelets and the aight and curved rods are shown to be artifact appearances caused by the action of lgi techniques on the lipochondria, &c.;
(*c*) irregular vacuoles of various sizes;
(*d*) yellow globules in some neurones, especially from old locusts.

Introduction

NERVE-CELLS are among the first cells to differentiate into their definitive form in insect embryos and they probably never divide after their st differentiation. It was thought, therefore, that they would be convenient aterial for a cytological study of differentiation and growth. However, in der to study the cytological changes during their early history it was cessary first to ascertain their structure in the adult.

Among the earlier contributions to the subject the most detailed study was at of Beams and King (1932). These authors worked on several species of thoptera. Their conclusions are in several respects at variance with my own d it will be convenient at the outset to mention their opinions as to the ture of the cytoplasmic inclusions. These opinions may be summarized as lows:

(1) The Golgi bodies of nerve-cells are separate elements 'consisting of a ttened plate-like osmiophobic medulla bordered by an osmiophilic rim'. In e view these bodies appear like rings, semicircles, or banana-shaped structes; in profile more or less as straight rods.

(2) 'There are no preformed red-staining granules or vacuoles in the nervel.' Neutral-red injections evoke formation of neutral-red bodies which are

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‘aggregated dye particles’. When ganglion cells are stained supravita vacuoles appear as the end-product of the catabolic action of the cell.

(3) Mitochondria are composed of granules, short rods, and filaments, a do not anastomose to give rise to the ‘Golgi net’ of Hosselet (1929).

(4) ‘A basket-like net of neurofibrillae surrounds the nucleus.’

(5) Intracellular trabeculae emerge from the fibrous capsule of the cell a penetrate the cytoplasm, as far as approximately one-third of the distance the nucleus. They are probably a supporting framework for the cells.

(6) No Nissl bodies are demonstrable in the cells.

The findings of other investigators will be mentioned at the appropri places below.

MATERIAL

Adults of *Locusta migratoria*, kindly supplied by the Anti-locust Resear Centre, London, were used for this work. In the ganglion, the associati neurones can easily be distinguished from the motor neurones in both fix and living material. The former are spheroidal, while the motor neuro are pear-shaped. These motor neurones are of very different sizes, their t diameters varying generally from 23–62 μ and 40–86 μ. They have a lar amount of cytoplasm in the cell, the nucleus being only 10·5–30 μ in diamet The motor neurones of the thoracic ganglia were chosen for study.

METHODS

Unfortunately there is no single method which would give reliable inf mation about all the cell-inclusions and it is dangerous to draw conclusio about the various constituents of the cells unless they are studied in a vari of ways. In this work, cell-inclusions were examined during life by t different methods mentioned below and an attempt was made to correl the appearances, as far as possible, with those obtained in fixed prepa tions.

For work with untreated living cells, a drop of locust blood was pipetted from a puncture made in the thorax, care being taken that it did not get mix with the crop-fluid which comes out of the mouth even with slight pressu on the animal during handling. In order to prepare the material, the locu were decapitated and the thoracic sternites quickly cut out of them. T ganglia could then easily be removed from the sternites, the whole dissecti taking a few minutes. The ganglia were teased in blood and the cells stud in a screw-compressor. The cells could be pressed to any desired extent wh watching them with the microscope. The phase-contrast apparatus used of the type described by Baker, Kempson, and Brunet (1949). The phase obj tive was the Watson fluorite 2 mm. For micro-dissection of the cells the sing control micromanipulator devised by Barer and Saunders-Singer (1948) used.

For vital staining janus green, janus black, dahlia, neutral red, nile blue, a methylene blue were tried. These dyes were used both as injections into emocoele and supravitally. The dilutions were done in the following saline lution:

sodium chloride,	10 per cent. aq.	10 vols.
calcium chloride,	10 per cent. aq.	0·2 ,,
distilled water		90 ,,

When a dye was to be injected it was used at a concentration of 0·25 per nt. in this saline medium. For supravital work the dyes were all used at 0·01 r cent.

The following methods were used for the fixation, impregnation, and stain-g of the ganglia:

(1) the osmium-impregnation technique of Kolatchev (1916) with Meves's d Champy's fluids as fixatives;

(2) the silver-impregnation technique of Aoyama (1929);

(3) Metzner's method for staining mitochondria (Metzner and Krause, 928) with Altmann's and Helly's fluids as fixatives. Baker's 'ISO' fixative, to e published shortly, was also used. This is an osmium mixture that preserves e shape of mitochondria well.

These techniques were applied to paraffin sections. Turpineol was found seful as an antemedium for paraffin embedding, as it did not harden the ssues much.

The following staining and colouring techniques were used:

(1) Sudan IV method for triglycerides.
(2) Baker's (1944) sudan black method for the Golgi element.
(3) Baker's (1946) acid-haematein test for phospholipines.

For these three methods the material was embedded in gelatin and cut on e freezing microtome.

Some ganglia were also fixed in 1 per cent. osmium tetroxide and embedded ithout staining in celloidin.

RESULTS

itochondria

The mitochondria of these cells are of two types—granular and filamentous gs. 1 and 2, A). The granular type are up to 0·4 μ in diameter and more umerous than the thinner filamentous type which are about 1·7 to 2·6 μ ng. Both kinds of mitochondria are easily seen by phase contrast and can e vitally stained by janus green B and janus black, the latter giving better sults, especially with filamentous mitochondria. In fixed preparations the ethods of Metzner, after fixation in the fluids mentioned above, show them uite well. No specialized distribution-pattern could be made out. In many lls, however, the filamentous mitochondria are seen to be more numerous und the nucleus. Usually they appear to be concentrated round the lipo-ondria mentioned below.

Filamentous mitochondria were often observed with swellings at their ti which might be due simply to local enlargement or else to the union o granular mitochondrion with the end of a filamentous one.

It may be pointed out here that when cells are observed by phase-contr microscopy, it sometimes appears at first glance that the nucleus is su rounded by a basket-like net of thin threads. By careful focusing, it can read be seen that the threads are independent filaments and do not join togeth

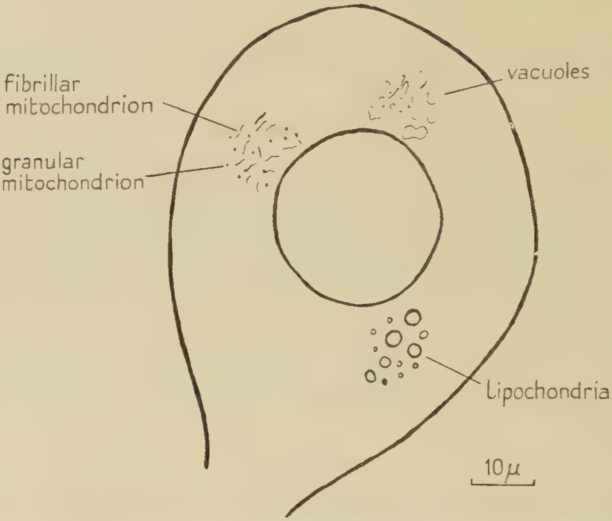

Fig. 1. A diagrammatic representation of the various cytoplasmic inclusions in the mo neurones of *Locusta migratoria*.

These are fibrillar mitochondria and should not be confused with 'neu fibrillae'.

Baker's osmiophil bodies and the Golgi controversy

Baker (1953) defines osmiophil bodies as objects which (1) unquestiona exist in the cytoplasm of the cell during life, (2) are blackened by the Go techniques, and (3) are not seen in routine microscopical preparations. Sim fat (triglyceride) globules are not included in this category.

There are no triglyceride globules in the neurones of *Locusta*. Sudan colours the fat-body surrounding the ganglia brilliantly, but leaves neurones untouched.

The following studies were made to find whether any osmiophil bodies defined above) could be detected in these cells.

(*a*) *Study by micro-dissection and phase-contrast microscopy*. To avoid a facts as far as possible the nerve-cells were examined in the insect's own bl by these two methods. When living cells are micro-dissected, globules spheroids of sizes varying from 0·4 μ to 2·6 μ are seen in the cytoplasm (1). These are loosely embedded in the cytoplasm and can easily be eject bserved under phase contrast these spheroids are not clearly visible in intact ells. The cells are thick and the refractive index of these bodies is not so arkedly different from the ground cytoplasm as is that of mitochondria. owever, if the living cell is well flattened under the coverslip of the comressor, so as to improve the phase-contrast image, they do show up quite learly. When cells are only gently pressed by the coverslip, clumpy areas are een here and there in the cytoplasm. These areas appear bluish by phase ontrast. When the pressure is increased, spheroids are seen exactly in the laces where the bluish areas were noticed before. The possibility that presure causes some material to separate from the ground cytoplasm cannot be ntertained, for the spheroids are clearly visible when the cells are ruptured by icro-dissection. When the cytoplasm is allowed to flow out of a cell into the urrounding blood or saline fluid, the spheroids float away as separate bodies ith very sharp outlines. By positive phase contrast they now appear to have black cortex and a bluish medulla.

(*b*) *Vital staining.* All the dyes tried acted very much better when injected han when applied supravitally. Not only do the spheroids stain more rightly, but the vacuolation effect, mentioned later on, was very much educed. Further, the nucleus took a slight tinge of the dye when used upravitally even in very low concentrations, while after injection the ucleus remained perfectly colourless.

In the cells examined after injections of neutral red, nile blue, and methyene blue, the spheroids colour brightly (fig. 2, B). The number of the spheroids hat take up the colour and the intensity of the colouring depend on the amount f the dye that penetrates the various cells. Thus in a teased and vitally tained ganglion it is possible to find many cells where no staining has ccurred, while others show very different numbers of coloured spheroids. In ttempts with injections of amounts varying up to lethal doses of the dyes, it as not found possible to colour all the spheroids. Further, the smaller spheroids ake up colour much more easily than the larger ones and appear as 'granules'.

Beams and King (1932) definitely state that the neutral-red bodies are not reformed structures, but simply aggregations of dye particles, their number arying with the amount of the dye injected and the time it was allowed to act. There is no doubt in my mind that this is a mistake: it is certainly the pheroids that segregate the vital dyes. Thus when cells stained with neutral ed are seen under phase contrast, the red colour is completely masked by the hase-picture, and one sees the spheroids as if they were not stained at all. The removal of the annular plate from in front of the light source reveals the tain in the spheroids again. It is, therefore, concluded that these neutral-red pheroids are a real constituent of the cytoplasm in the locust's neurones.

(*c*) *Have the spheroids a cortex?* It was mentioned above that when the pheroids have flowed out of the cell into a fluid of lower refractive index than he cytoplasm, they appear, by positive phase-contrast microscopy, to have a ark peripheral region or cortex. This cannot in itself serve as a proof of real eterogeneity, for it might well be an optical 'edge effect' familiar to workers with phase contrast. A similar rim is sometimes seen by ordinary microscop in sections stained with Metzner's method; in this case the apparent cortex red with acid fuchsin while the centre is almost colourless. It will be reco lected that Fischer showed long ago (1899) that mixtures of acid fuchsin an picric acid are capable of giving precisely this appearance to granules th are in fact homogeneous. Sudan-black preparations also show no division int cortex and medulla.

It was not found possible to decide whether the spheroids are truly homo geneous or not. Fischer (1899) made a careful study of what he calle *Ringgranula* produced by the action of fixatives on bodies known to be homo geneous. Since phase-contrast microscopy, certain fixatives, and dyes used i pairs, can all give a false impression of a cortex and medulla when the obje under study is in fact homogeneous, the utmost reserve is necessary in drawin a conclusion on this subject.

Smallwood and Rogers (1908), referring to the spheroids in the nerve-cell of gastropods, remarked: 'Considerable work has been done to determine th question whether or not these cytoplasmic vacuoles have a definite wall. A ver critical study of the cytoplasm in contact with the vacuoles fails to show an evidence of a separate wall. The vacuoles in the living nerve-cell forcibl remind one of the food vacuoles in Protozoa which appear to have a wall, bu this appearance is really due to contact of fluids of different refractive inde We believe it is no more proper to speak of a wall for these vacuoles than it i to say that the numerous vacuoles in Protozoans have walls.'

It is unfortunate that many recent students of cytoplasmic inclusions hav not been so critical as were Smallwood and Rogers 45 years ago.

(*d*) *The Golgi controversy*. Osmium techniques (Kolatchev) show ring semicircles, straight, curved, and irregular rods in the cells (fig. 2, C and D Aoyama's silver method also shows them, though the osmium preparatio are clearer. These bodies, according to Beams and King, are separate Gol bodies, each consisting of a flattened plate-like osmiophobe medulla bordere by an osmiophil rim seen in various views. Bowen (1920) has described simil 'osmiophil platelets' in the spermatocytes of Hemiptera, and they have bee frequently recorded in various cells. The following facts are relevant in th consideration of the living counterpart of these Golgi appearances.

FIG. 2 (plate). All the photomicrographs are at the same magnification.

A, a medium-sized motor neurone of *Locusta*, vitally stained with janus black to show t granular and fibrillar mitochondria. A few of the smaller lipochondria have also taken up t stain.

B, a large motor neurone intravitally stained with neutral red to show lipochondria various sizes.

C, a neurone prepared by Kolatchev's method to show the osmiophil platelets. The ganglic was fixed in Champy's fluid and osmicated for 4 days at 37° C.; 6 μ section.

D, same as C, except that Meves's fluid was used as fixative.

E, a neurone as seen in a sudan-black preparation. The ganglion was fixed in Champy's flu and osmicated for only one day. The platelets could just be seen in the sections, which we then treated with sudan black; this coloured them strongly.

The spheroids are osmiophil. If cells in a hanging drop are exposed to the vapour of osmium tetroxide from a drop of the solution in the bottom of a cavity-slide, black cortices appear on the spheroids. Cells put in a drop of osmium tetroxide solution show the same appearance. Further, when Meves-fixed material was crushed on a slide, blackened spheroids were seen as a cell-fragment fell apart. In the same way, after a few days' osmication further blackening of the spheroids was seen. Some material was fixed in different fixatives (Meves and Champy) and osmicated at different periods and temperatures. Different amounts of osmium could be deposited under different conditions of osmication, and the clearest preparations were obtained by osmication of Champy-fixed material for four days.

It is therefore believed that the spheroids are responsible for the Golgi appearances. Further, the 'osmiophil cortex' is mostly, if not entirely, an osmium deposit, formed artificially on the surface by the addition of reduced particles to those previously deposited. And, as has already been mentioned, the thickness of this 'cortex' varies with the degree of osmication, as can be seen in the photographs (fig. 2, C and D) of the Golgi preparations. Both of these were osmicated for 4 days, the difference being in fixation only. The 'osmiophobe medulla', about which there are such various opinions in the literature (it has indeed been regarded as an optical illusion (Weiner, 1930)), represents the spheroid itself. The various rod-shapes are sectional views of the platelets and some are probably also produced by the joining up of the smaller spheroids. The smaller spheroids in the living cells are very irregularly thrown together—in rows, in small clumps, and also round the larger spheroids—and it may be that they are responsible for many irregular shapes in the Golgi preparations.

The Golgi techniques are not specific or reliable and it could be claimed that many irregular appearances are unspecific deposits of osmium or that the mitochondria get clumped together into rods. In this respect Metzner and Hirschler preparations, especially the former, give a much more faithful picture of the spheroids of all sizes. These, especially the smaller ones, can also be seen in ganglia simply fixed in 1 per cent. osmium tetroxide and embedded in celloidin. The flat and distorted shape of 'platelets' is the effect of the reagents of the standard Golgi techniques. They also appear somewhat bigger, and this may be due partly to deposit on the surface, or, as Covell and Scott (1928) found, partly to actual swelling.

Workers have reached very different conclusions about these 'osmiophil platelets'. Beams and King (1932), as has already been mentioned, interpret the 'osmiophil cortex' of the platelets as the homologue of the classical Golgi apparatus and the 'osmiophobe medulla' according to them 'apparently has no homologue in the classical Golgi apparatus'. The homology of their 'cortex' with the classical Golgi apparatus is only applicable if the latter is also regarded as an impregnation artifact. It is somewhat surprising that while the above authors studied both living and fixed cells, they apparently did not investigate the living counterpart of the 'discrete Golgi bodies'.

Monti (1915) described a delicate Golgi network in the arthropod nerve-cell resembling the structure in vertebrate neurones which, according to her, ha been overlooked by other workers. Hosselet (1929) also describes a networ in insect nerve-cells formed by the hypertrophy of mitochondria. Beam and King (1932) ascribe the descriptions of these authors to neurofibrillae In the present work networks or hypertrophied mitochondria were neve seen and it is difficult to understand the findings of the above-mentione authors.

(*e*) *Concluding remarks.* From the above study the author believes that th 'discrete Golgi bodies' or the 'osmiophil platelets' are the larger spheroids artificially modified by the osmium techniques. The main difficulty in comparing the spheroids with the Golgi preparations would appear to be that th spheroids, especially the larger ones, are not very readily coloured by neutra red. In many cells only the smaller spheroids colour (as seen in the diagram given by Beams and King) and there is an obvious disparity in size and th number of the two bodies. However, examination of the living cells by othe methods and successful neutral-red preparations (see fig. 2, B) should leave on in no doubt about the true state of affairs.

The spheroids appear to be lipoidal in constitution. They take up suda black and also give a positive result with Baker's acid-haematein test. That i is the platelets which give a positive result with the above two tests was show by the following method, which is similar to Thomas's (1947). Ganglia wer fixed in Champy's fluid and osmicated for one day only. The osmiophil platelets could be just made out in sections. These were then treated with suda black and the platelets were strongly coloured by it (fig. 2, E).

In this connexion it is instructive to recall Baker's opinion about the platelets. In his recent contribution to the Golgi problem (1953) he said: 'Il se peu que leur forme soit sphérique pendant la vie, mais qu'elles soient aplaties pa les réactifs utilisés dans la technique. S'il en est ainsi, elles représenteraien une variété spéciale de lipochondries.'

It does seem reasonable to describe these osmiophil bodies of the *Locust* neurone as lipochondria of various sizes. They show the same characteristic as lipochondria in general. Thus they are osmiophil as well as sudanophil an can be stained by acid fuchsin and by haematoxylin when preceded by 'ISO or Hirschler fixatives. The only special feature of these lipochondria appear to be that they are perhaps not as readily visible in the living cell as in som other animals and tissues.

There are a few important points which should be noted in connexion wit these bodies. The smaller lipochondria deserve special mention. They resemble the larger ones in their refractive index and their ability to segregat neutral red, but they also stain with janus dyes (fig. 2, A); and, though of lowe refractive index than mitochondria, in matter of size they form a graded serie with the granular mitochondria. It would thus appear that they are intermediate forms between mitochondria and the larger lipochondria. Further they do not show up as such in Golgi preparations, and it may be suggeste tentatively that they get clumped together into straight or curved rods. However, they do appear as separate granules in ISO-Metzner preparations.

Vacuoles and vacuolation

Dying neurones were observed to become highly vacuolated. Cells when allowed to stand in neutral-red solution for half an hour also developed a large number of very lightly staining vacuoles. Dustin (1947) showed reversible vacuolation of the red blood-cells of Amphibia, birds, &c., after injection of ammonium salts. Grzycki (1953) observed large numbers of vacuoles staining lightly with neutral red in ganglion-cells in snails when the animals were kept in water at 40–45° C. for 20–30 minutes.

However, vacuoles could be seen in many neurones examined as quickly as possible and in the locust's own blood (fig. 1). It appears that they occur normally in the cells or else are produced by the teasing of the ganglion. The vacuoles give the impression that they are watery exudates from the cytoplasm. They appear not to be invariably present; they are irregular in shape; they only take up a tinge of neutral red and cannot be stained in any other way, nor isolated.

It should be noted that the lipochondria when out of focus in the microscope appear as clear vacuoles. The two constituents, however, are entirely different bodies. When the cells are broken the lipochondria float away as separate bodies, while the vacuoles remain in the cytoplasm. Further, the refractive index of the vacuoles is lower than that of the lipochondria. They always appear as clear spaces without any appearance of a cortex. They are mainly concentrated round the nucleus, giving it a patchy appearance, and on the periphery of the cells. They are often seen to merge with one another. They are not osmiophil, though they show up better when the ground cytoplasm becomes grey on exposure to the vapour of osmium tetroxide. Many empty spaces seen in Zenker, Hirschler, and osmium preparations (fig. 2, D), called lymph spaces by Smallwood and Rogers, probably represent vacuolated peripheral areas of the cell.

The earlier workers did not distinguish clearly between the vacuoles and lipochondria. Hodge (1892) studied changes in the nerve-cells of honey bees under normal fatigue by examining them in the morning and after their day's work. Besides decrease in nuclear size he reports vacuolation of the cytoplasm as a change that is due to fatigue. From his diagrams it would appear that he is referring to what are here called vacuoles. Smallwood and Rogers however discuss Hodge's vacuolation under their heading of the objects that are homologized with lipochondria in the present paper.

Yellow globules

Especially in cells from old locusts, besides the above-mentioned inclusions, globules of distinctly yellow colour are often seen. They do not stain with neutral red, though from shape and general appearance they appear to be modified lipochondria. They are clearly visible in ordinary transmitted light and probably belong to the category of 'yellow pigment granules' or 'wear an tear pigment' described in nerve-cells of many other groups.

Intracellular trabeculae and neurofibrillae

Beams and King describe their 'trabeculae' as a supporting framework fo the cells, formed of fibres which have penetrated from the capsule of the cells The neurofibrillae are believed to be a network surrounding the nucleus Neither of these could be seen in the living cells. Whether there is an ultra structure of molecular pattern responsible for these appearances is a problem of submicroscopic morphology.

The work was done under the supervision of Dr. J. R. Baker and I have a deep sense of gratitude to him for teaching me methods of microscopy and micro-technique, and for guidance all through the work. It is a pleasant duty to thank Professor A. C. Hardy for accommodating me in the Department of Zoology and Comparative Anatomy at Oxford. I also thank Professor G. C. Varley and Dr. B. M. Hobby for much helpful advice and encouragement.

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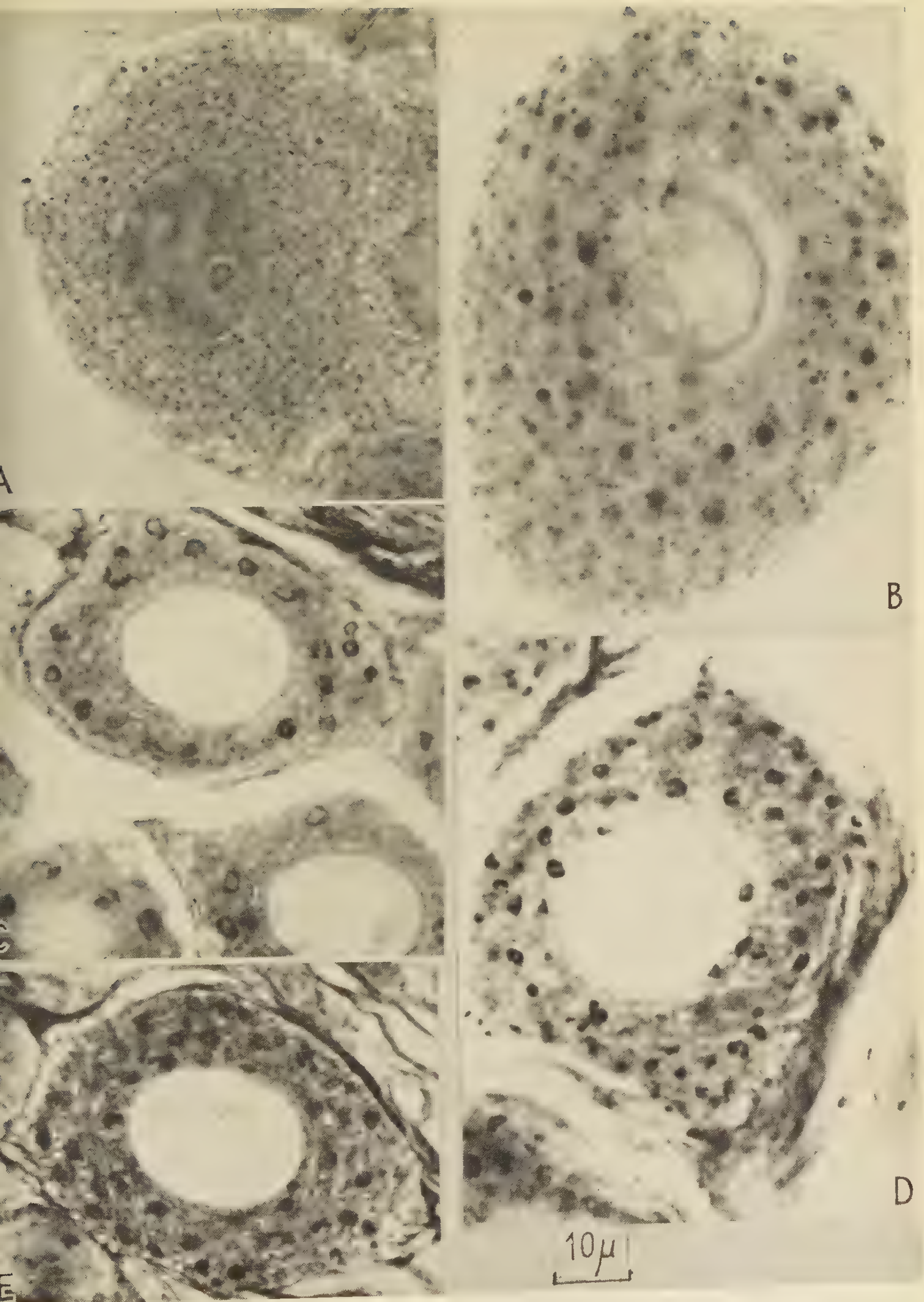

FIG. 2—S. A. SHAFIQ

The Sarcosomes of Heart Muscle

Their Isolation, Structure, and Behaviour under various Conditions

By K. W. CLELAND AND E. C. SLATER

(*From the Department of Zoology and the Molteno Institute, Cambridge*)

With two plates (figs. 1 and 3)

SUMMARY

1. A study of the distribution of the granules in rat heart-muscle fibres confirmed
e early morphological findings. We prefer to describe these granules under the name
sarcosomes, first introduced by Retzius.

2. In sections, heart sarcosomes are seen as rods in close association with the aniso-
opic disks of the myofibrils. The rod-shape is believed to be due to compression
the sarcosomes between adjacent myofibrils. Isolated sarcosomes which retain the
asticity of those in the living state would be expected to be spherical.

3. Preparations of sarcosomes isolated with hypertonic sucrose contained many
ds. Spheres were obtained with isotonic saline or sucrose. Water, ethylene glycol,
glycerol gave greatly swollen and otherwise altered sarcosomes.

4. In hypotonic media, sarcosomes underwent a series of partially reversible
orphological changes termed 'transformation'. These changes, which are caused by
netration of water into the sarcosomes, are described in detail.

5. Studies of 'transformation' gave clear evidence that the intact sarcosome is com-
sed of a central body in the form of a gel surrounded by a membrane. As the
nicity of the medium was decreased, the body progressively disappeared, leaving
entually a large vesicle surrounded by the membrane. In this condition the mem-
ane readily breaks up into particles which contain all the enzymes which constitute
e respiratory chain, but is devoid of most dehydrogenases and phosphorylating
zymes.

6. On standing in isotonic media the membrane is rapidly damaged, causing
netration of solutes so that the sarcosomes transform in much the same way as in
potonic media. This 'spontaneous transformation' was greatly delayed by the
lcium-chelating agents versene or citrate and by adenosine diphosphate or triphos-
ate, and was accelerated by calcium.

INTRODUCTION

THIS paper is concerned with an investigation, carried out in conjunction with biochemical studies (Cleland and Slater, 1953; Slater and Cleland,
52, 1953, *a*, *b*), of the morphology of the sarcosomes, the respiratory granules
the heart muscle of vertebrates. An examination of the sarcosomes *in situ*
nfirmed extensive but not widely known studies made between 1841 and
16. Our biochemical work required the isolation from heart muscle of a
spension of sarcosomes resembling as closely as possible the granules in
intact muscle. The values of different suspending media for this purpose
compared in this paper. Studies of the behaviour of isolated sarcosomes

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in hypotonic media and of the factors affecting their morphological stabil are also included; these studies have given some information about structure of sarcosomes.

The presence of granules in the sarcoplasm of striated muscle has be known for more than a century. Henle (1841) was the first to see them, muscle treated with acetic acid, while the first detailed study was made Kölliker (1856; 1888, *a*, *b*; 1889), who recognized their wide distribution the animal kingdom. Aubert (1853) was the first to describe the unusua large and abundant granules of insect muscle.

In 1888 Kölliker separated the granules from insect muscle and studi the effect of various treatments on the morphology of the isolated granul This must be one of the earliest isolations of an intracellular inclusion. particular interest is his description of the effect of hypotonic conditio 'The granules swell extraordinarily in water and change into vesicles wi a clear but delicate membrane. During this process the contents assume t form of a half-moon lying to one side and undergo a partial solution.'

The presence of sarcosomes in a wide range of muscles became mo evident from the work of Retzius (1890) and Knoll (1891). Holmgren (191 and Bullard (1913, 1916) studied the granules in the heart muscle of a co siderable number of species. They found that the majority of the granul were at the level of the A(anisotropic)-bands of the fibres and that sm numbers were present at either pole of the nucleus. Bullard described the as soft, plastic lipoprotein bodies which assumed a wide variety of shapes.

After 1916 little attention was given to these granules until very recentl Much of our knowledge of the cytochrome system has come from Keilin study of particulate preparations from heart muscle (e.g. Keilin and Hartre 1940), but the origin of these particles in the muscle was not studied. With the last few years it has been recognized that the respiratory activity of t muscle is located in the sarcosomes, which in this respect correspond to t mitochondria in other tissues. This was first shown by Watanabe and Willia (1951) for insect muscle and by Harman and Feigelson (1952, *a*, *b*, *c*) a Cleland and Slater (1953) for heart muscle. In the latter paper we have sho that the heart-muscle preparations used by Keilin and Hartree are obtain by the disintegration of the muscle granules; this process is examined further detail in this paper. Kisch and Bardet (1951) and Perry and Hor (1952) have studied the granules of heart muscle and skeletal muscle respe tively with the electron microscope.

Nomenclature

In the early literature the granules of striated muscle have been referred as 'granules' (Henle, 1841), 'interstitial granules' (Kölliker, 1856; Holmgr 1910; Bullard, 1913, 1916), 'sarcosomes' (Retzius, 1890), or 'mitochond (Regaud, 1909; Duesberg, 1910; Luna, 1913). Holmgren distinguish between Q(A)- and J(I)-interstitial granules, according to their relation to muscle striations. The identification of the granules with mitochondria

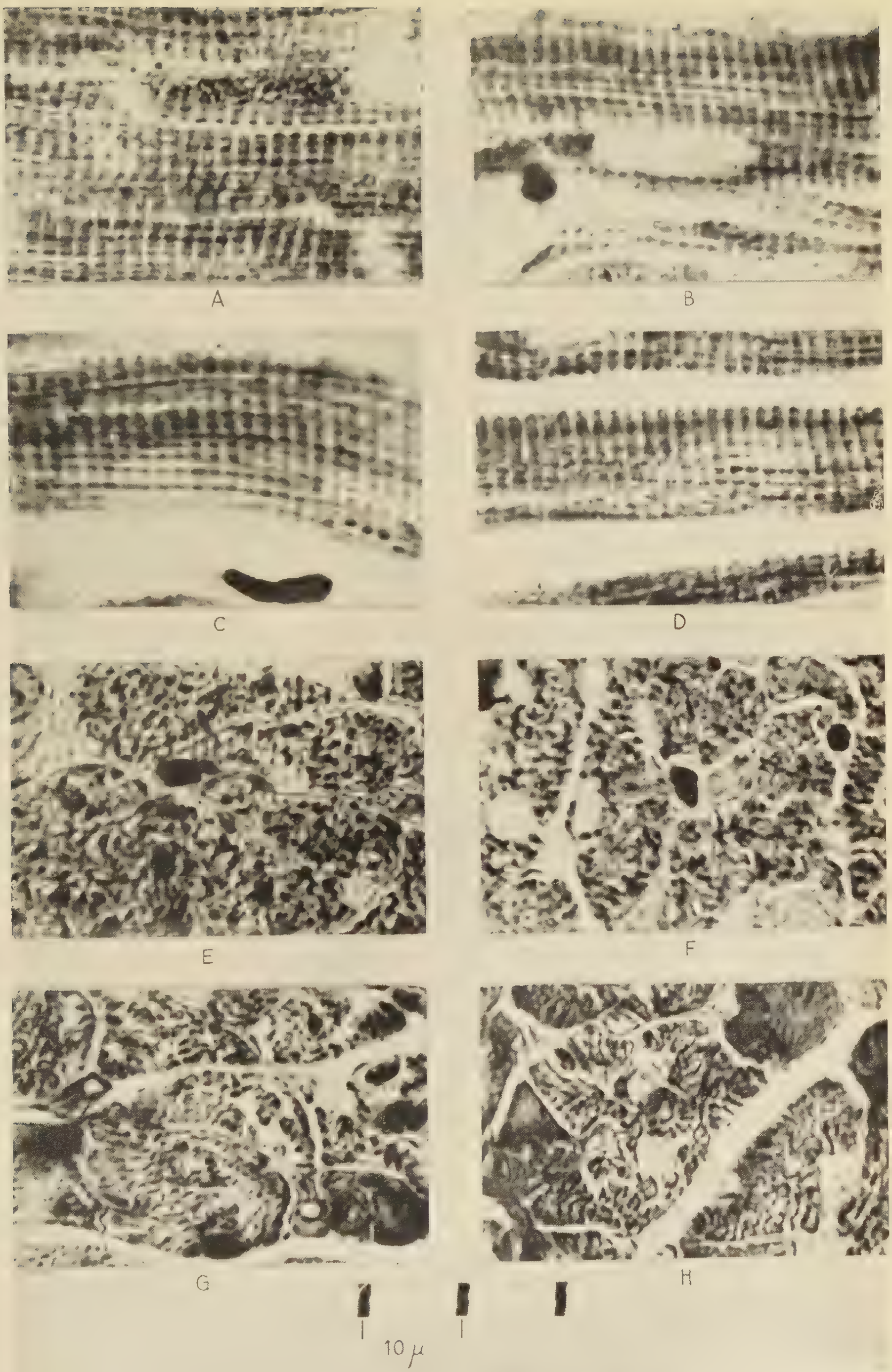

Fig. 1

K. W. CLELAND AND E. C. SLATER

ıde by Regaud, Duesberg, and Luna on the basis of staining reactions and supported by the recent biochemical studies, mentioned above.

Although it is now clear that the function of these granules in muscles rresponds to that of mitochondria in other tissues, we consider that to refer them as mitochondria would obscure certain important differences. In rticular, heart-muscle granules are distributed in a very regular manner the muscle and do not change position like mitochondria in other cells. We efer the term 'sarcosome' (Retzius, 1890); it is cytologically non-comittal and conforms to the nomenclature of other parts of the muscle fibre.

METHODS

Except where the contrary is stated, the sarcosomes were prepared from e hearts of adult rats, of either sex and of various strains, by the method scribed elsewhere (Cleland and Slater, 1953). Briefly, the method consists grinding chopped heart with sand in a suitable suspending medium and bjecting the resulting homogenate to differential centrifugation at 0–5° C. ıe low-speed centrifugation (700 g for 3·5 min.) removes the myofibrils, nuc-, and connective tissue, and the sarcosomes are then sedimented by higher eeds (7,500 g for 7·5 min.) and washed once with fresh suspending medium.

MORPHOLOGY OF HEART MUSCLE

xed sectioned material

The most satisfactory demonstration of sarcosomes in paraffin sections (5 μ) ıs made by staining with Altmann's aniline/acid fuchsin. The heart muscle s fixed in Helly's fluid (24 hours) and then treated with 2·5 per cent. tassium dichromate for 24 hours at 37° C. (Baker, 1945).

Longitudinal sections are shown in fig. 1, A–D. The sarcosomes make up out one-eighth to one-quarter of the total area of the fibre.

Two groups of sarcosomes may be recognized—the perinuclear and the ermyofibrillar sarcosomes. The former, which occur in the perinuclear coplasm at either pole of the nucleus, constitute only about 1 per cent. of total sarcosomes and are mainly spheres. The intermyofibrillar sarcones, which occur in the sarcoplasm between the myofibrils, are blunt rods in ost areas and are distributed regularly in both the longitudinal and transverse es of the fibres (see fig. 2 for a diagrammatic representation). A direct nonstration that these transverse rows of sarcosomes lie at the level of the striations was possible by polarization microscopy of favourable areas. In ıer areas, dark contraction bands or Z-bands could be seen between adjaıt transverse rows, again indicating that the rows themselves lay at the level of A-bands. Examination under polarized light also indicated that the longituıal rows of sarcosomes were determined by the close relation between sarcones and myofibrils and that these two components are separate. Breaks in continuity of the rows regularly occur at the intercalated disks.

In transverse sections, shown in fig. 1, E–H, the morphology of the sarcones is more variable. In many areas they are spherical or oval, while in other areas are seen rods which are often bent and have what appears to a complex form. By careful focusing these complex forms could usually resolved into a number of single rods, lying one above the other. In ar

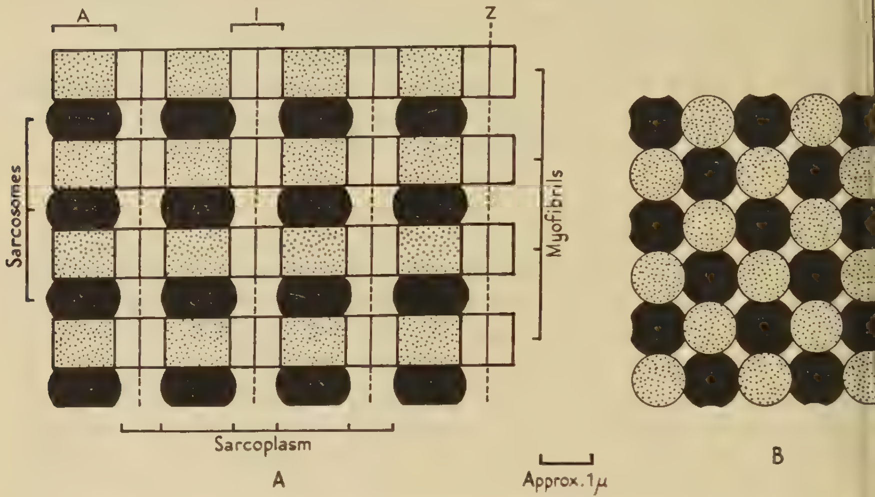

FIG. 2. Diagrammatic representation of the relation between sarcosomes and myofibrils, co structed on the assumption that the distribution of sarcosomes is completely regular and tl the myofibrils are all of the same diameter. A, longitudinal view of a portion of a fibre which there is moderate compression of the sarcosomes. B, transverse view of a porti showing slight compression of the sarcosomes. The sarcosomes are black, the A-disks stippl

where the myofibrils are stained sufficiently to be clearly visible, the bent ro lie between adjacent myofibrils, curved round the periphery of one of the

Sarcosomes could also be demonstrated after formol-calcium fixati (Baker, 1946), embedding in Aquax (G. T. Gurr), and staining with saturat aqueous cresyl violet (Grübler). These sections, mounted in glycerol, show the same distribution of sarcosomes as in the Altmann preparations, but t sarcosomes were spheres instead of blunt rods. There was considerably l shrinkage with this method.

The sarcosomes of heart-muscle are so abundant and so regularly d tributed that careful consideration had to be given to the possibility that Altmann procedure was staining the A-disks of the myofibrils, not the sar somes. This was excluded by the following considerations: (1) the appeara

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FIG. 1. Altmann preparations of sectioned rat heart muscle. The large bodies are red bl corpuscles. A and B, longitudinal sections of fibres showing rod-shaped intermyofibri sarcosomes and spherical perinuclear sarcosomes. In the right lower corner of A is an in calated disk, free of sarcosomes. C and D, longitudinal sections showing the sarcosomes c fined to the A-bands. In D some of the sarcosomes are a little displaced from their nor positions. E and F, transverse sections of areas showing mainly spherical sarcosomes. G an transverse sections of areas showing rod-shaped sarcosomes. The apparently complex sh of some of the sarcosomes in these areas is largely due to overlapping of several sarcoso as could be seen by careful focusing.

sarcosomes in transverse sections; (2) demonstration of the spatial separa-
n of the two components by polarization microscopy; (3) the proportion
the total area staining with the Altmann procedure is about the same as
e percentage of the total proteins of the muscle located in the sarcosomes
pproximately 20 per cent., Cleland and Slater, 1953); (4) colouring methods
ich would be expected to be more specific for sarcosomes, e.g. the sudan
ack method of Baker (1946), showed bodies similar in shape and distribu-
n to those obtained with the less specific Altmann procedure.

esh and fixed unsectioned material

Examination by dark-ground, phase-contrast, and ordinary microscopy of
ased and lightly ground fresh heart failed to reveal the intermyofibrillar
rcosomes clearly, although spherical perinuclear sarcosomes could some-
nes be seen. Refractile, elongated rods could be seen in heart teased or
ound in 0·88 M sucrose; blunt rods were seen less clearly in 0·44 M potas-
um chloride. In both solutions the perinuclear sarcosomes were spherical.

Satisfactory staining of the sarcosomes, either free or in their normal place
the tissue, could not be obtained with vital dyes (cresyl violet, methylene
ue, brilliant cresyl blue, neutral red, janus green, nile blue—all from G. T.
urr) applied to muscle teased in Tyrode's solution or 0·88 M sucrose.

Blackened, mainly spherical sarcosomes arranged in the typical manner
ere seen after fixing teased fibres in osmium tetroxide.

ISOLATION OF SARCOSOMES

For the disintegration of the tissue before differential centrifugation we
ve used the old method of grinding with sand rather than the more modern
ethods of tissue disintegration, which are liable to fragment the myofibrils,
ereby making more difficult the separation of sarcosomes from myofibrils.
ve layers were recognized in the sediment from the low-speed centrifuga-
n (3·5 min. at 700 g) of the ground muscle. First (at the bottom of the
be) was coarse sand, then a thick grey layer of long fibre-fragments (and
nd), followed by another thick layer of white fibre-fragments (and sand),
thin layer of red blood corpuscles, and a thin layer of very fine sand-frag-
ents matted together with white, sticky amorphous material, probably of
yofibrillar origin. The sand grinding did not liberate all the sarcosomes,
considerable proportion of which were found within the relatively long
agments of muscle fibre; few were in the layer of white fibre-fragments.
grinding of this sediment with more sand only slightly increased the yield
the sarcosomes and was not normally adopted. It is probable that the
sidual sarcosomes were firmly compressed between the myofibrils, since,
dging by the absence of the colour of myoglobin in the fibres, most of the
rcoplasm had already been extruded. Those sarcosomes which were
erated during the initial grinding were probably extruded with the tide
sarcoplasm; the remainder would be freed only on complete disintegration
the fibre.

A comparison of the range of sizes of isolated sarcosomes with that sarcosomes in the intact muscle (compare fig. 1 and fig. 3, A–D) strong suggests that some fragmentation of the granules occurs during the isolatio Because of the contiguity of sarcosomes and myofibrils and the presence the tough sarcolemma investing the fibres, some fragmentation of the granu during isolation is probably unavoidable. The degree of fragmentation ma however, be increased considerably by more prolonged grinding with san When the muscle is lightly homogenized with an apparatus of the Potter ty there is considerably more fragmentation than in the usual homogenat obtained by grinding with sand.

Choice of suspending medium

In a search for the most suitable suspending medium a number of solutio were examined. Observations were made on the appearance of the sarcosom immediately after isolation and on the changes undergone during exposu to room temperature. Both unfixed and fixed (osmium tetroxide, final co centration 1 per cent.) sarcosomes were studied. The pH of all solutions w between 7 and 7·5.

Non-electrolytes. Hypertonic sucrose (0·88 M), recommended by Hogboom, Schneider, and Pallade (1948) for the preservation of the rod-sha of liver mitochondria, yielded a mixture of about 30 per cent. rods and 70 p cent. spheres. Isotonic sucrose (0·33 M) gave only spheres. Hyperton (0·88 M) or isotonic (0·33 M) glycerol or ethylene glycol gave greatly swoll 'transformed' sarcosomes (see p. 335), which were very similar to tho obtained when distilled water was used as suspending medium. Since transformation was caused by adding glycerol or ethylene glycol to a suspe sion of sarcosomes in isotonic saline the phenomena observed with the compounds is due to their rapid penetration into the sarcosomes. The la of osmotic effect of glycerol and ethylene glycol has already been observ by Lehninger and Kennedy (1948) with liver mitochondria, using a bi chemical criterion of hypotonicity.

Electrolytes. Isotonic potassium chloride (0·165 M), isotonic potassiu chloride/magnesium chloride mixture (4:1, based on the cation compositi of heart (Lowry, 1943)), 'phosphate-saline' (0·043 M Sörensen phospha pH 7·3; 0·051 M KCl); 'saline' (0·02 M Sörensen phosphate, 0·135 M K adjusted to pH 7·4) and isotonic potassium citrate (0·0825 M) all gave sphe (fig. 3, A–D). Comparison of these preparations with one another and with is tonic sucrose by means of a comparison eyepiece did not reveal any gr morphological differences except that those prepared with 'phosphate-sali were slightly larger and those with citrate a little smaller. Hypertonic potassi chloride (0·44 M and 0·6 M) gave a small proportion of oval sarcosom

Sarcoplasm. Heart sarcoplasm was prepared by pressing minced ho heart in a hydraulic press under a pressure of 1 ton/in.^{2} The first press jui mostly blood, was discarded and the remainder neutralized and clarified centrifugation at 15,000 g for 20 minutes. Sarcosomes isolated in this medi

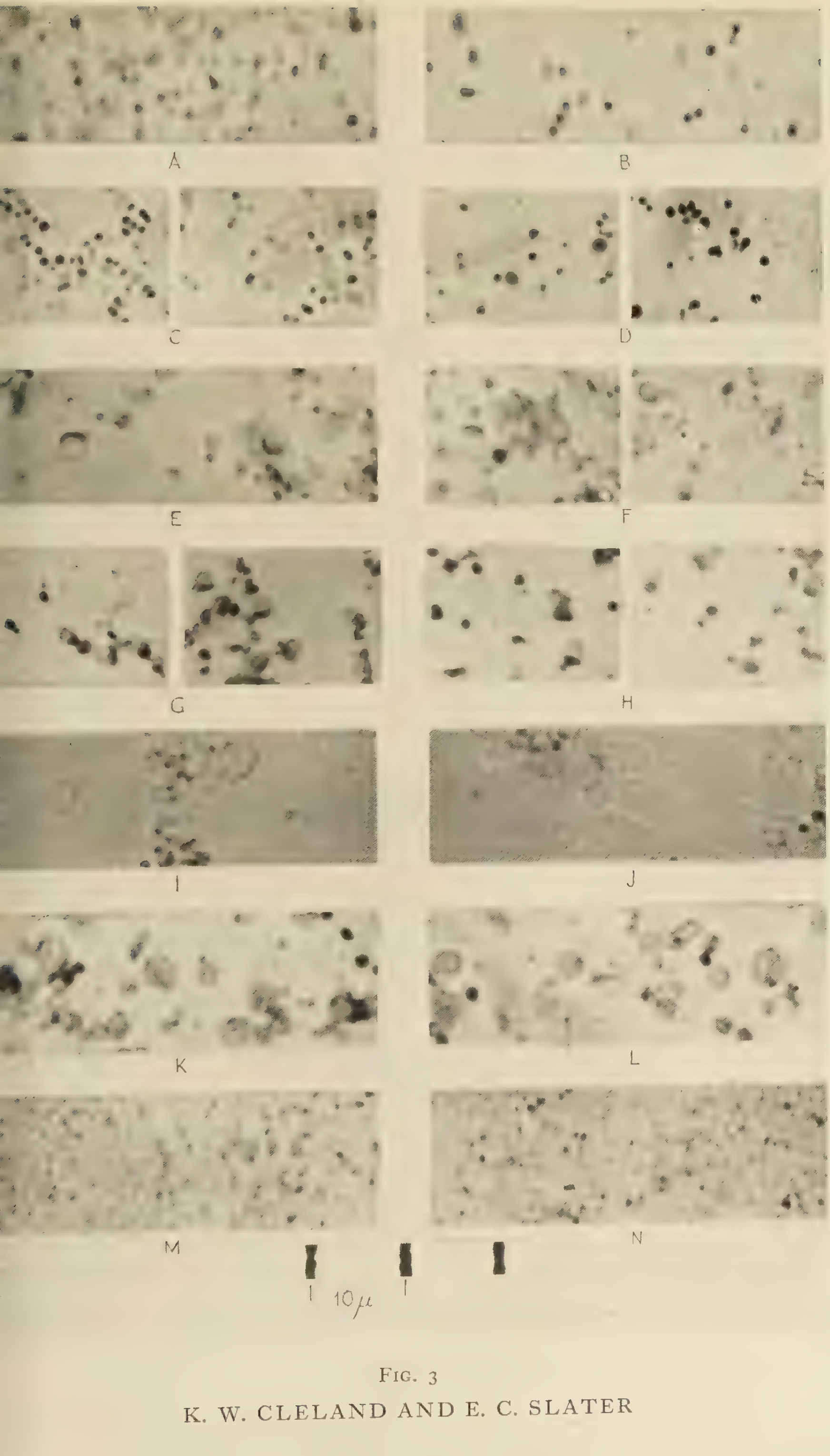

Fig. 3

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ere spheres indistinguishable from those obtained with isotonic electrolytes.

Calcium-chelating media. Sarcosomes prepared in all the above media ransformed’ rapidly when allowed to stand at room temperature. The most able were those isolated in citrate or sucrose, while ‘phosphate-saline’ ielded the least stable preparations.

The relative stability of citrate preparations suggested that calcium, present a concentration of $1\cdot5 \times 10^{-3}$ M in heart (Slater and Cleland, 1953; Cleland, 953), might be concerned with the instability in other media. Calcium is nown to promote the breakdown of the pigment granules in sea-urchin eggs Heilbrunn, 1930) and to have a pronounced effect on the respiration of omogenates of sea-urchin eggs (Hultin, 1950; Cleland and Rothschild, 952). Accordingly, we studied the effect of adding versene (ethylenediamine etra-acetic acid), a very effective chelating agent for calcium (Schwarzenbach nd Ackermann, 1947), to saline media. An isotonic solution containing ·01 M versene, 0·02 M phosphate, 0·115 M KCl, adjusted to pH 7·4, gave arcosomes which, although initially indistinguishable from those made in aline, were very much more stable than those made in isotonic sucrose.

TRANSFORMATION OF SARCOSOMES

When heart-muscle sarcosomes are suspended in hypotonic media, in media containing penetrating substances, or even, after a time, in isotonic media devoid of chelating agents for Ca^{++}, they undergo a series of structural changes resulting in the formation of the ‘half-moon bodies’, first studied by Kölliker (1888*b*) in isolated insect sarcosomes. More recently Zollinger (1948) and Walker (1952) have described the formation of crescents in mitochondria n the liver cell, and Harman (1950) and Walker (1952) have studied the same process in isolated liver mitochondria. Similar changes have been frequently noted in mitochondria present in tissue culture cells under hypotonic conditions (e.g. Hogue, 1919; Anitschkow, 1923).

We shall refer to these morphological changes as ‘transformation’. Harman (1950) has already referred to agents which bring about morphological changes as ‘transforming agents’, but he uses the word ‘transform’ in a biochemical sense, thus: ‘a series of chemical agents and physical conditions which transform the conjugated pyridino-protein enzymes of the cyclophorase complex into dissociated, classical form.’ We use the word with a purely morphological, not a biochemical, connotation.

Transformation may be brought about in two distinct ways: (i) by suspending the sarcosomes in hypotonic media (or in media containing a substance which penetrates the sarcosomal membrane); (ii) by allowing the sarcosomes to stand at room temperature. The first method, which will be called *hypotonic transformation*, is caused by the movement of water into the sarcosomes and is practically instantaneous. The second method, referred to as *spontaneous transformation*, is due to movement of solute and water into the sarcosome; it is considerably slower.

At different stages of the transformation, either hypotonic or spontaneous, four different morphological types of granule were recognized, namel (1) spheres formed from rods obtained with hypertonic media; (2) crescent (3) vesicles; (4) fragmented vesicles.

After the various treatments described below, the sarcosomes were fix in osmium tetroxide (final concentration, 1 per cent.); no change other tha enhanced visibility was demonstrable after the fixation. The structure of th fixed transformed granules was more clearly seen with the ordinary micr scope by the use of critical illumination than with the phase-microscop Smears of fixed granules stained with a saturated aqueous solution of cres violet and mounted in glycerol gave confirmatory and supplementary evidenc about the internal structure, despite considerable distortion of the structu by the drying.

Hypotonic transformation

To obviate the spontaneous transformation which otherwise occurs o standing, the experiments described in this section were carried out in th presence of versene. Photomicrographs are shown in fig. 3, and a diagrammatic representation of the changes is given in fig. 4.

Rods to spheres. When the tonicity of the medium is reduced from 0·8 osmolar to about 0·5 osmolar, the rods (fig. 4, A) obtained with 0·88 M sucros or the ovoids obtained with 0·44 M KCl change into spheres (fig. 3, A–D fig. 4, B). (The osmolar concentration is the total concentration of all th molecular or ionic species, calculated on the assumption that all are osmotically active and that salts are completely ionized.)

Crescents. When a suspension of granules in isotonic saline is diluted wit water, the first change is a considerable swelling accompanied by a corresponding decrease of refractility (fig. 4, C). This swelling, which is reversible increases with decreasing tonicity to 0·05–0·06 osmolar, below which a furthe change occurs. This consists of the separation of the sarcosome into tw parts—a crescent-shaped, osmiophil, refractile body and a complementary

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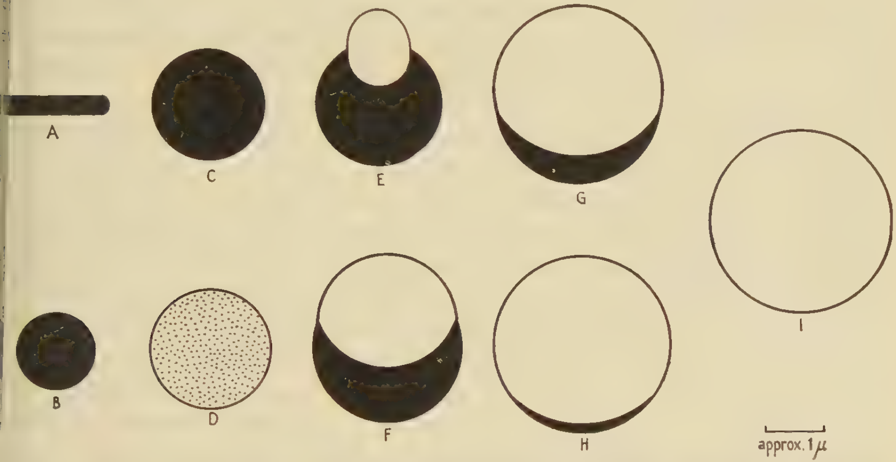
FIG. 3. Preparations of isolated sarcosomes fixed in osmium tetroxide (final concentration 1 per cent.). Both unstained suspensions and dried smears stained in cresyl violet and mounte in glycerol are shown. A and B, sarcosomes suspended in isotonic saline (0·02 M phosphate 0·135 M KCl, pH 7·4). The contours are somewhat blurred because of Brownian movement C and D, stained smears of the sarcosomes shown in A and B. E and F, spontaneously transformed sarcosomes, showing crescents. Membrane, body, and vesicle are clearly visible. I some cases the body appears to be outside the membrane, but careful focusing shows tha this is not the case. About the middle of the upper edge of E are some swollen sarcosome which have not transformed into crescents; these also show a clear membrane. There ar also some almost normal sarcosomes in the suspension. G and H, stained smears of the transformed sarcosomes shown in E and F. Although there has been shrinkage and distortion durin drying, the stained membrane is easily visible. I and J, suspensions of vesicles in distille water. The body present in E and F has disappeared, leaving only the membrane enclosin the vesicle. Some fragmentation of the vesicle has occurred. K and L, stained smears of th vesicles shown in I and J. Although distorted the membrane is clearly seen, often wrinkled The dark material in the vesicle is the dried solution of body material. In L, the vesicles wer partially fragmented by mechanical agitation; the resulting particles are to be compared wit the Keilin & Hartree preparation shown in M and N. M and N, stained smears of the Keili & Hartree preparation, showing the two types of granules described by Cleland and Slater (1953

itially bullous, non-refractile, non-osmiophil portion with a common interce bounding both parts. The two parts will be called the 'body' and 'vesicle' spectively and the common interface the 'membrane'. These changes are ustrated in fig. 3, E–H, and fig. 4, E–H. The membrane has also been seen swollen sarcosomes as a denser cortical investment of the poorly refractile dy (fig. 3, E; fig. 4, D). In cresyl-violet stained smears of granules in which e swelling has not proceeded too far, the membrane is seen as a clearly efined chromophil line of regular thickness bounding the vesicle (fig. 3, G, H).



IG. 4. Hypotonic transformation of the sarcosomes. A, rods in 0·88 M sucrose. B, C, spheres 0·5 osmolar and 0·05 osmolar solutions respectively. D, the membrane as it sometimes ppears in swollen sarcosomes which have not formed crescents. E, F, G, H, progressive disppearance of body as the tonicity is decreased from 0·05 to 0·01 osmolar. I, vesicles below 0·01 osmolar.

s the tonicity is decreased below 0·05–0·06 osmolar, the vesicle increases, hile the body decreases in volume and becomes elongated. An upper limit the increase in vesicle size exists at approximately 0·01 osmolar, although ther changes in the body occur at lower tonicities.

Sarcosomes prepared in isotonic sucrose transform into crescents at the ame or slightly lower tonicities (approx. 0·04 M sucrose). Between 0·05 and ·02 osmolar, the volume of the vesicle in these preparations is less than in line preparations suspended in saline, but the final volumes attained at out 0·01 osmolar are about the same. The probable explanation of these ifferences is that potassium chloride but not sucrose is able to penetrate to me extent when the membrane is stretched. Sarcosomes prepared in hypernic (0·88 osmolar) sucrose or saline transform into crescents at a higher nicity (0·15 osmolar), probably owing to injury to the sarcosomal membrane d penetration of solute from the hypertonic medium.

Vesicles. At tonicities lower than about 0·01 osmolar, a further stage of e transformation occurs. The crescent-shaped body completely disappears, leaving only the vesicle bounded by the membrane (fig. 3, I–L; fig. These vesicles are no longer turgid and may be indented by centri packing. Preparations of vesicles are best obtained by using distilled w throughout the isolation procedure. Isotonic or hypertonic glycer ethylene glycol yield similar preparations, except for occasionally incom loss of body.

Fragmented vesicles. Sarcosomes in the late stages of crescent forma and particularly those which consist of vesicle alone, are fragile and m fragment when the suspension is vigorously shaken (fig. 3, L). Mecha agitation is necessary to bring about this fragmentation, which does not o when the transformed sarcosomes are merely left at room temperature an hour or two. Microscopic examination, which may be misleading in bo of this size, suggests that the fragments are in the form of minute sph which are either single or joined together in clumps or strings. The membr evidently breaks in several places at once.

These fragments are microscopically indistinguishable from the parti of the Keilin and Hartree heart-muscle preparation (fig. 3, M and N), wh appear in this form immediately on release from the heart muscle. The Ke and Hartree preparation is made by mincing heart muscle obtained from slaughter-house (it may be some hours old), and then washing for sev hours with changes of tap water (this contains about 0·0015 M calcium Cambridge). The washed mince is then ground with sand in dilute phosph buffer and the resulting small particles collected by differential centrifugat or acidification. It is clear from this paper that the sarcosomes in mus subjected to prolonged treatment with water containing calcium would h changed into vesicles. If not already fragmented, squeezing them between myofibrils during the sand grinding would be an efficient way of promot fragmentation.

Reversibility of hypotonic transformation. Transformation from rods i spheres is irreversible; restoring the original hypertonicity produces c shrunken spheres.

The transformation into crescents is largely reversible, if too low a toni has not been used. A return to isotonicity after a period at 0·03–0·05 osm does not, however, yield a suspension identical with the original suspens There are a number of smaller spheres and an occasional crescent in addit to spheres of normal size. The small spheres are probably due to a los internal osmotic components through the stretched membrane. The p viously isotonic medium will then be hypertonic with respect to the inte contents of the granule, even when the latter has shrunk to normal s further shrinkage will therefore occur. When sarcosomes which have b transformed to crescents and returned to isotonic media are again expose hypotonic conditions, the second transformation does not usually procee far as the first. This is probably also due to loss of internal osmotic c ponents during the first transformation as well as to permanent change the permeability of the membrane caused by stretching.

Sarcosomes which have been transformed at the lower tonicities where here is considerable loss of substance in the body or where it has disappeared ltogether do not revert to their original form when returned to isotonic media lthough the volume may decrease somewhat, the greatly stretched membrane f these sarcosomes is now evidently freely permeable to external solutes.

Spontaneous transformation

Suspensions of sarcosomes in isotonic saline relatively rapidly transform nto crescents on standing at room temperature. The rate of this spontaneous ransformation is slower in isotonic sucrose (cf. Knocke, 1909) and in isotonic itrate and is very slow when the sarcosomes are prepared and suspended in sotonic saline containing 0·01 M versene.

Spontaneously transformed granules are morphologically indistinguishable rom those which have undergone hypotonic transformation into crescents at bout 0·03 osmolar. However, three differences between hypotonic and sponaneous transformation were observed: (1) the later stages of the transformaion into crescents and the complete loss of body are not common; (2) sponaneously transformed sarcosomes show a marked tendency to agglutinate, and uspensions of such granules fixed in osmium tetroxide are recognizable by he rapid settling of the aggregates; (3) spontaneous transformation is only parially and temporarily reversed by increasing the tonicity to 0·64 osmolar.

Spontaneous transformation is probably caused by an injury to the sarcoomal membrane which makes it more readily penetrated by solutes. The bsence of the later stages observed in hypotonic transformation is due to loss of internal osmotic constituents, while the temporary nature of the eversal is due to the irreversible damage to the membrane accompanied by reater permeability to solutes. The tendency of these granules to aggregate s also probably a reflection of changes in the membrane.

Factors promoting spontaneous transformation. Spontaneous transformation s considerably accelerated by decreasing the tonicity of the suspending nedium. For example, in one experiment the times required for 50 per cent. f the sarcosomes to transform in 0·16, 0·13, and 0·10 M KCl were 25, 20, nd 10 minutes respectively. This is probably because the damaged memrane is more permeable when stretched.

The rate of transformation was increased by the surface-active agents odium oleate (0·01 M) and desoxycholate (0·1 per cent.). A higher conentration of desoxycholate (1 per cent.) caused the complete disappearance f all microscopically visible structures. The rate was also increased by rsenite (0·015 M) but not by dinitrophenol (0·001 M).

The addition of Ca^{++} (0·01 M) to saline suspensions caused a slight but efinite increase in the rate of transformation. This effect of Ca^{++} was further tudied with sarcosomes which had been prepared in saline-versene and ubsequently washed twice with versene-free saline. These preparations, lthough free of versene, contain much less Ca^{++} than normal preparations Slater and Cleland, 1952, 1953*b*) and, correspondingly, are much more

stable. In fact, the rate of transformation of such versene-washed preparatio
is only slightly greater than that of preparations suspended in saline-verse
The addition of 0·01 M Ca^{++} to versene-washed preparations greatly increas
the transformation rate to a value near that obtained with normal saline p
parations. These experiments strongly suggest that the rapid transformati
of normal preparations is due to the calcium they contain.

The temperature coefficient of spontaneous transformation is high;
17° C. sarcosomes transform about 15 times faster than at 2° C.

Factors promoting stability. The most striking increase of stability can
achieved by the addition of the calcium-chelating agent, versene (see p. 33

It was found that the reaction mixture used in biochemical experimen
on oxidative phosphorylation (Slater and Cleland, 1953*a*) also had a ve
marked stabilizing effect. This mixture contains α-ketoglutarate, phospha
adenosine diphosphate (ADP), adenosine monophosphate (AMP), magnesiu
fluoride, and malonate. Omitting the substrate (α-ketoglutarate) had no effe
while omitting the ADP removed practically all the protection. When test
alone, both ADP and ATP (adenosine triphosphate), but not AMP, had
marked stabilizing effect. Dinitrophenol (0·001 M) did not diminish the pr
tection given by ADP; this was therefore not due to oxidative phosphoryl
tion of ADP to ATP coupled with the oxidation of residual substrate
However, some ATP would be formed from the ADP by the action
myokinase present in the sarcosomal preparation (Cleland and Slater, 195

Discussion

Modern histological text-books and recent reviews of muscle structu
(e.g. Barer, 1948) pay little attention to the sarcosomes. In view of the
great physiological importance, some statement about their occurrence a
distribution, as recorded in the literature reviewed in the Introduction,
desirable. On the basis of their granules, striated muscles may be divid
into two classes which correspond to two physiological types.

Muscles with granules at the level of the I(isotropic)-bands are those wi
intermittent activity such as the skeletal muscle of vertebrates and invert
brates; their metabolism is often largely anaerobic during activity. The
I-granules are described as small spheres often lying paired in the sarcoplas
in close relation to the I-disks on either side of the Z-disk. There may
a tenuous thread passing between adjacent A(anisotropic)-disks and co
necting the I-granules of the same sarcomere.

Muscles with granules at the level of the A-bands are found in situatio
requiring sustained activity, such as the flight muscles of birds, bats, a
insects, and the heart muscle of vertebrates. These muscles, which are p
dominantly aerobic in metabolism, were shown by Keilin (1925) to be t
tissues with the highest cytochrome content. The A-granules are larger a
more numerous than the I-granules in the first muscle type, are usually o
or rod-shaped in tissue sections, and are confined to the sarcoplasm lyi
between adjacent A-disks and to aggregations at each pole of the nucle

hese perinuclear sarcosomes are spherical. In the flight muscles of insects he granules may be larger than in vertebrate heart muscle, are spherical, nd are not so obviously associated with the A-disks.

The description in this paper of fixed sections of heart muscle is in comlete agreement with the early literature. We cannot agree with the recent iew of Harman and Feigelson (1952*b*) that the sarcosomes (or mitochondria) re irregularly distributed and may even be present with their long axes ransverse to the muscle fibre. Harman and Feigelson's observations were made n material which had been fragmented in the Waring Blendor. Since sarcoomes are extruded with the sarcoplasm, such material could hardly be xpected to retain the regular arrangement characteristic of the intact fibre.

Recent work summarized in the Introduction has made it clear that the unction of the A-sarcosomes is the all-important one of carrying out the espiratory metabolism of the cell and generating the ATP which appears to e involved in the mechanism of action of the myofibril. Whether there is ny particular significance in the close proximity of the sarcosome to the -disk of the myofibril remains to be seen. The recent papers of Paul and perling (1952) and Chappell and Perry (1952) indicate that the small and parse I-granules of skeletal muscle have essentially the same function as the rger and more numerous A-granules in heart muscle. It is not surprising hat those muscles which show the greatest functional activity should have he highest respiratory metabolism and the highest concentration of granules. Iowever, the significance of the different cytological distribution of the arcosomes in the two types of muscle is not clear.

It is widely believed (however, see Barer, 1948) that the Z-disks of the yofibrils are joined with one another and with the sarcolemma by transverse onnexions bridging the sarcoplasm. This is supported by Kisch and Bardet's 1951) electron micrographs of sections of heart muscle. If this is so, each arcosome must be enclosed in a pocket limited by the Z-membrane. The igh yield of sarcosomes obtained by sand grinding, which merely breaks the bres into fragments of various lengths, indicates that the Z-connexions must e broken by the grinding. This is interesting since the transverse striations re not disarranged even in small fibre fragments freed from sarcosomes. his shows that the disk apposition in the transverse axis of the fibre does ot require intact Z-connexions.

solated sarcosomes

Shape. Neither this study nor the previous work has determined the shape f the intermyofibrillar sarcosomes in the intact living fibre. The isolation of ods in hypertonic sucrose might be thought to indicate that the sarcosomes the fibre are rods, since Hogeboom *et al.* (1948) believe that this medium etains the rod-like shape of liver mitochondria. However, sarcosomes isolated ith hypertonic sucrose have a very low respiratory activity (Slater and leland, 1953*a*) and it seems doubtful if this would be true of sarcosomes the intact cell.

The opinion has often been expressed (e.g. Hogeboom and others, 194
Harman, 1950; Harman and Feigelson, 1952, *a*, *b*, *c*) that hypertonic med
are required to preserve the rod-shape of mitochondria because the norm
intracellular tonicity is very much higher than that of blood. Robinson (195
has suggested, on the basis of the swelling of non-respiring kidney slices
isotonic media, that the intracellular contents are approximately 0·55–0·
osmolar. However, this is not supported by direct analyses of the inorgan
constituents of tissues (e.g. see the tabulation of Davies and Krebs, 195
Lowry (1943) and Cleland (1953) have analysed rat heart muscle for cation
the osmolar concentration in the aqueous phase of the muscle, calculate
from the cation content and assuming a mean anion valency of 1·7, is 0·3
(Lowry) and 0·320 (Cleland). The osmolar concentration in the aqueous pha
of beef heart muscle, calculated in a similar manner from the cation valu
of Wilkins (1934), is 0·256. Thus, if the fibre contents are greatly hyperton
they must contain a very high concentration of an unionized solute of sm
molecular weight. It would be surprising if such a substance has escape
detection.

If, as seems probable, the fibre contents are approximately isotonic wi
blood, it would be expected that sarcosomes in the fibre would be spheric
unless some constraint were put upon them. In fact, in both living a
sectioned muscle, the perinuclear sarcosomes are spherical. The intermy
fibrillar sarcosomes, however, are in close apposition to adjacent myofibr
which change in diameter during contraction. This suggests that the sha
of these sarcosomes in the fibre will be determined by the degree of co
pression to which they are subjected by the myofibrils. The partially reversib
changes of form which occur during transformation show that the sarc
somes are reversibly deformable. Some evidence was obtained that the sha
of the sarcosomes does depend upon the degree of compression betwe
myofibrils. Thus, in Altmann preparations, where shrinkage was appreciab
the sarcosomes were rods of variable length, while in formol/calcium/cres
violet preparations, or in teased muscle fixed with osmium tetroxide (whe
there was considerably less shrinkage), the sarcosomes were mostly spheric
In hypertonic media, where because of withdrawal of water the myofibr
would be more closely packed, the sarcosomes were even more elongat
than in the Altmann preparations.

Once the constraint was removed, it would be expected that sarcosom
would become spherical. We consider, therefore, that the rods obtained wi
hypertonic sucrose are evidence that the normal physical properties of t
sarcosomes are altered by the withdrawal of water so that instead of becomi
spherical they retain the rod-form impressed upon them in the cell by the my
fibrils. Dilution of hypertonic sucrose (0·88 M) preparations to about 0·45
sucrose destroys the rods, presumably because the consequent rehydrati
of the sarcosomes restores their elasticity. It is significant that neither the
sarcosomes nor those isolated with isotonic media change into rods when t
medium is made 0·88 osmolar.

In a series of papers published after the present work was completed, Harman and Feigelson (1952, *a*, *c*) have described a 'target' form of sarcosome. It probably corresponds to the form illustrated in fig. 4, D of this paper. In heir latest paper Harman and Feigelson (1952*c*) apparently consider that his 'target' form is more normal than the 'spherical dense' form (presumably what we have represented in fig. 4, B). Our finding that unaltered sarcosomes are as shown in fig. 4, B is more in line with the earlier conclusion of these authors (Harman and Feigelson, 1952*a*), although earlier still (Harman, 1950) the normal form was thought to be rods.

'Body' of sarcosome. The observations on the transformation of rat heart sarcosomes clearly indicate that these granules consist of two parts—a central body surrounded by a peripheral membrane or cortex.

The body, which is in a gel state, is capable of only limited swelling, down to tonicities of 0·06 osmolar, beyond which the cohesive forces between the membrane and body are exceeded by the elastic tension in the swelling body so that the body separates from the membrane over a limited area and becomes crescent-shaped. With decreasing tonicity the body diminishes in volume and, eventually, at very low salt concentrations it disappears. Since phase-contrast microscopy indicates that the refractive index of the vesicle contents is considerably higher than that of the suspending medium, it is very probable that the body has dissolved in the vesicle contents.

Membrane. Kölliker (1888*b*) was the first to describe a thin but distinct membrane in water-treated sarcosomes of insect flight muscle. Much more recently, Claude (1944) concluded that the swelling and disintegration of solated mitochondria in water showed the existence of a membrane, while Zollinger (1948) described a membrane on mitochondria in intact cells treated with hypotonic solutions. Electron micrographs have also given some evidence of a membrane in liver mitochondria (Claude and Fullam, 1945; Dalton, Kahler, Kelly, Lloyd, and Striebich, 1949) and in skeletal-muscle sarcosomes Perry and Horne, 1952). In our experience, the membrane is much more obvious by direct microscopical examination of sarcosome suspensions than by using dried smears.

There is strong evidence that the sarcosome membrane is semi-permeable. Firstly, the great swelling of sarcosomes in media of graded hypotonicity is egular, apart from the complication introduced by the transformation; econdly, the swelling is largely reversible; and thirdly, this paper and the more quantitative studies described elsewhere (Cleland, 1952) provide strong evidence for the selective permeability of different substances; for example, glycerol and ethylene glycol penetrate very rapidly, and potassium chloride penetrates more rapidly than sucrose.

Harman (1950) denies the existence of a semi-permeable membrane in mitochondria, which he regards as tightly coiled or compact gels without membrane (see also Green, 1952). It is difficult to reconcile this concept with he properties of sarcosomes (and mitochondria) summarized in the above paragraph. Moreover, it is not at all clear how such a gel could give rise to the characteristic crescent-shaped granules, with a clearly marked interfa between the vesicle and the external fluid, described by Kölliker and in th paper. Harman (1950) described in detail the course of the transformatio from spheres to crescents, but did not recognize the membrane. Accordingl his interpretation of these changes differs considerably from that given in th paper. He considers that the absence of lysis in de-ionized water and th non-selective penetration of radioactive ions are substantial evidence again the membrane hypothesis. With regard to the first point, we find that th granules do in fact undergo what corresponds to lysis when the great stretched membrane is mechanically damaged. We cannot regard Harman radioactive tracer experiments as conclusive for the following reasons. (1) Th preparation used was a kidney 'cyclophorase' preparation, which consists the total sedimentable material of homogenates; this means that a consider able proportion of the preparation would consist of loosely packed inter tubular connective tissue, basement membrane, &c., which would not hav semi-permeable properties. (2) After treatment with radioactive materia Harman collected the residue by a centrifugation of only 500 g for 5 minutes the interstitial fluid must have been considerable. (3) Even assuming that th technique used did measure the permeability of the mitochondria, it possible that an exchange of external (radioactive) cation for internal (nor radioactive) cation occurred, without any movement of anion. The exper ments on light-scattering of sarcosomes reported elsewhere (Cleland, 195 suggest that the sarcosomes are, in fact, relatively permeable to cations, bu show different degrees of permeability to anions.

There is some biochemical evidence that might appear to support the ide that sarcosomes do not possess a membrane. These granules react rapidl with either reduced or oxidized cytochrome *c*, which, being a protein, woul not be expected to penetrate a biological membrane. However, evidence h been presented elsewhere that the system of enzymes which constitute th respiratory chain (diaphorase, succinic dehydrogenase, and the cytochrom system) are situated in the membrane (Cleland and Slater, 1953). It is likel then, that added cytochrome *c* reacts with proteins in the membrane an does not have to penetrate into the body of the sarcosome. Further, mar substrates which are oxidized by sarcosome suspensions, e.g. succinat fumarate, and malate, do not penetrate the sarcosome and must, presumabl react with enzymes in the membrane. It is possible that the sarcosomal bod contains those enzymes, e.g. phosphorylating enzymes, which are not four in preparations of the membrane (Keilin and Hartree heart-muscle prepar tions). However, the available information does not allow us to exclude th possibility that the sarcosomal body is merely an inactive core about whic an enzymically active surface is stretched.

The membrane must be quite strong, since it shows very little deformatio when the attached body is stretched along the inner surface of the membra (fig. 4, H). Furthermore, it can be greatly extended without rupture, althoug in this state it is fragile and agitation of the suspension causes it to bre nto small granules. If stretched beyond a certain limit the membrane does ot return to its original surface area when the internal osmotic pressure is ecreased by loss of internal osmotic constituents or exceeded by increasing ne tonicity of the external medium. Thus only the earlier stages of the ormation of crescents are reversible, and after the body has completely disppeared the greatly stretched membrane is relatively flaccid. Even moderate tretching of the membrane causes an increased permeability to solutes.

In common with other biological membranes such as the membrane of the ed blood corpuscle (Parpart and Dzieman, 1940), the Keilin and Hartree reparation contains a large amount of lipids (30–40 per cent. of the dry reight, Slater, 1949; Cleland, 1953).

pontaneous transformation

Spontaneous transformation is caused by an increased permeability of the arcosomal membrane to the solute of the suspending medium. Associated rith this increased permeability is a loss of ability of the sarcosomes to xidize α-ketoglutarate and to bring about oxidative phosphorylation (Slater nd Cleland, 1952, 1953*b*). The following considerations make it unlikely hat the morphological changes are responsible for the loss of enzymic ctivity. (1) Crescents appear after the loss of enzymic activity, while mere welling of the granules without crescent formation causes increased, not ecreased, enzymic activity (Slater and Cleland, 1953*a*). (2) Granules susended in isotonic sucrose transform more slowly than those suspended in aline, but lose their enzymic activity at the same rate. (3) Lowering the onicity increases the transformation rate, but not the loss of enzymic activity.

There is, however, some relation between the morphological and biohemical phenomena, because certain treatments influence both in a parallel ray. For example, both the spontaneous transformation and the enzymic nactivation are greatly slowed by versene, ATP, or ADP. It seems likely that common factor, possibly the level of some energy-rich phosphorus comound in the sarcosome, determines the onset of both morphological and iochemical changes. When this compound is exhausted by processes which nvolve calcium, irreversible changes are initiated in the structure of the arcosomal membrane and in the complex of enzymes which make up the -ketoglutaric oxidase system. According to this hypothesis, versene would ct by inhibiting some calcium-activated mechanism responsible for the estruction of the endogenous energy-rich phosphorus compound. This natter is further discussed elsewhere (Slater and Cleland, 1953*b*).

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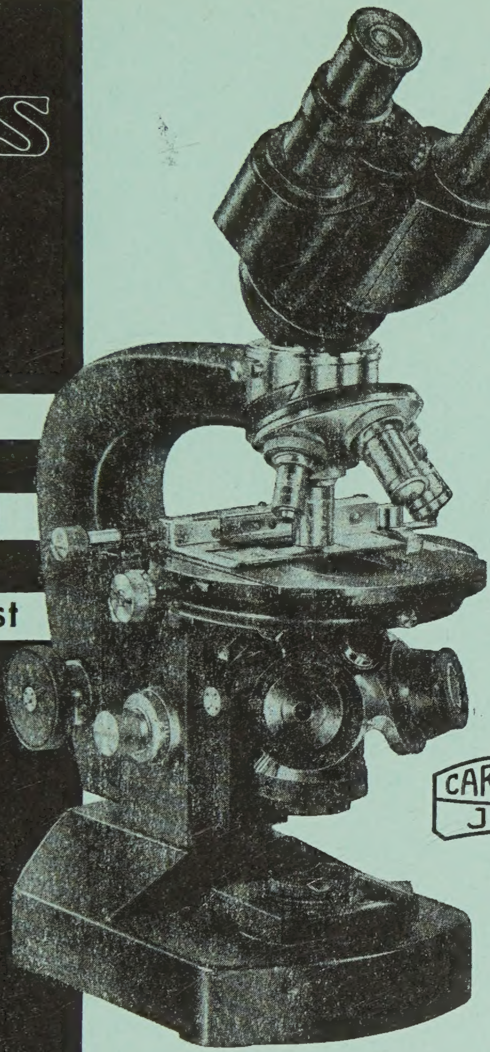

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